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Autoimmune antibodies anti-AchR as biomarkers in Myasthenia Gravis

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To my parents

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"We are going to find out what went wrong. And then we will try again, and we will fail again. Because that is what progress looks like"

Unkown

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Abstract

Myasthenia Gravis (MG) is an autoimmune disease triggered by antibodies directed against proteins involved in cell signaling at the neuromuscular junctions, resulting in failure of the neuromuscular transmission and muscle weakness (Conti-Fine *et al.*, 2006). The majority of these autoantibodies (≈85%) are directed to the nicotinic acetylcholine receptor (anti-AchR antibodies) present in skeletal muscle cells, but the exact area of the receptor that is targeted is still not known (Tzartos *et al.*, 1991). There is however a significant group of patients that do not express these antibodies. This heterogeneity may be associated with different characteristics regarding the specific target, the affinity or avidity of the antibodies and it may account for different clinical phenotypes. Furthermore, due to the non-specificity of the drugs currently available, some patients respond poorly or not respond at all to treatment. Recently, patients with MG have been treated with Rituximab, a drug that targets CD20 positive cells, (a membrane associated phosphoprotein present in the surface of every B-cell, except stem cells, pro-B-cells and effector B-cells), and resulting in the complete depletion of circulating B-cells. However, not all patients with MG respond to this therapy.

This study aimed at determining what is the specific molecular target that is bound by the pathogenic antibodies and whether these antibodies are produced by CD20-positive B cells. We started by investigating a specific region of 10-amino acid sequence (WNPDDYGGVK) corresponding to the residues 67-76 of the extracellular domain of alpha1 subunit of AchR that has been suggested to be involved as a potential target, in an animal model of MG. Simultaneously the extracellular domain of the alpha1 subunit of acetylcholine receptor (CHRNA1) was synthesized by molecular cloning, to allow the subsequent isolation of different peptides and determination of their potential role as targets.

Serum samples from patients with MG were collected and tested for IgG antibodies that recognized this specific region of 10-amino acid sequence and CHRNA1 cloned protein, by enzyme-like immunosorbent assay (ELISA). Furthermore we intended to identify whether patients had CD20+ B lymphocytes involved in the production of these pathogenic antibodies, using flow cytometry. All patients gave informed consent before entering the study.

We showed that the serum from seropositive patients for the AchR did not recognize the WNPDDYGGVK synthetic peptide, which suggests that the MIR peptide described to be the main immunogenic region in antibody targeting in animal models of the disease (EAMG), might not be the same in humans. However, patients with MG seropositive for the AchR had antibodies directed towards the CHRNA1 suggesting that this subunit might be the main targeted region in this disease. The detection of CD20+ B lymphocytes involved in the production of these pathogenic antibodies can provide an easy tool to identify patients that might respond to anti-CD20 drugs, such as rituximab.

Keywords: Autoantibodies; Antibodies anti-AchR; Myasthenia Gravis.

Resumo

A Miastenia Gravis é uma doença auto-imune caracterizada pela presença de autoanticorpos que reconhecem proteínas envolvidas na sinalização neuromuscular, desencadeando falhas na transmissão neuromuscular e fraqueza muscular (Conti-Fine *et al.*, 2006). Maioritariamente estes anticorpos (≈85%) apresentam uma elevada afinidade para o receptor nicotínico de acetilcolina (anticorpos anti-AchR) presente nas células musculares esqueléticas (Tzartos *et al.*, 1991). Devido à falta de especificidade dos fármacos disponíveis actualmente, alguns doentes respondem de forma incompleta, ou não respondem de todo, ao tratamento. Recentemente, alguns doentes com MG iniciaram tratamento com Rituximab, um fármaco que tem como alvo o CD20, uma fosfoproteína associada à membrana celular presente na superfície de todas as células B (excepto as células estaminais, células pró-B e as células B de memória), o que resulta na depleção das células B circulantes. No entanto, nem todos os doentes respondem de forma significativa a este tratamento.

Este estudo teve como objectivo investigar quais poderiam ser as potenciais regiões antigénicas no receptor nicotínico de acetilcolina (AchR). Começámos por investigar uma região específica de 10 aminoácidos (WNPDDYGGVK) que corresponde aos resíduos 67-76 do domínio extracelular da subunidade alfa 1 do AchR. Simultaneamente, o domínio extracelular da subunidade alfa 1 do receptor de acetilcolina (CHRNA1) foi sintetizado por clonagem.

Todos os doentes assinaram um consentimento informado antes de entrar no estudo. Amostras de soro de doentes com MG foram usadas para testar a existência de anticorpos IgG que possam reconhecer a sequência específica de 10 aminoácidos e também a proteína CHRNA1 clonada, por ensaio imuno-enzimático (ELISA). Além disso pretendeu-se identificar, por citometria de fluxo, se os linfócitos B envolvidos na produção destes anticorpos patogénicos são CD20+.

O desenvolvimento de um método de ELISA específico mostrou que os doentes com anticorpos anti-AchR não apresentavam títulos de anticorpos que reconhecessem o péptido sintético WNPDDYGGVK, o que sugere que o péptido MIR descrito como a principal região imunogénica para os anticorpos em modelos animais da doença (EAMG), pode não ser o mesmo em seres humanos. Os resultados obtidos

pressupõem a existência de outras regiões antigénicas no AchR nicotínico. Na sequência da identificação dos alvos específicos correctos, a detecção de linfócitos B CD20+ envolvidos na produção destes anticorpos patogénicos pode proporcionar uma ferramenta fácil de identificar doentes que possam responder a fármacos anti-CD20, como o Rituximab.

Palavras-chave: Autoanticorpos; Anticorpos anti-AchR; Miastenia Gravis.

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Abbreviations

Aa	Amino acid
ABTS	2,2'-Azino-Bis(3-eThylbenzthiazoline-6-Sulphonic acid)
AchR	Nicotinic Acetylcholine Receptor
Asn	Asparagine
Asp	Aspartic acid
BCR	B-Cell Receptor
BIC	Bicarbonate
BL21 (DE3)	Bacterial *E.coli* strain
bp	Base Pairs
BSA	Bovine Serum Albumin
CAPS	Cryopyrin-Associated Periodic Syndromes
CHRNA1	Extracellular domain of Alpha1 subunit of Nicotinic acetylCholine Receptor
Cys	Cysteine
DEAE-dextran transfection	DiEthylAminoEthyl-dextran transfection
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	DiMethylSulfOxide
DNA	Deoxyribonucleic Acid
EAMG	Experimental Autoimmune Myasthenia Gravis
EDTA	EthyleneDiamineTetraacetic Acid
ELISA	Enzyme-Linked ImmunoabSorbent Assay
FBS	Fetal Bovine Serum
FSC	Forward Scatter Channel
Gly	Glycine
HBS	Hepes Buffered Saline

HCl	Hydrochloric acid
HEK-293T cells	Human Embryonic Kidney cells
HRP	HorseRadish Peroxidase
Ig	Immunoglobulin
IgG	Immunoglobulin G
IgM	Immunoglobulin M
Ile	Isoleucine
IL2ss	InterLeukin 2 signal sequence
IPTG	IsoPropyl β-D-1-ThioGalactopyranoside
IVIg	IntraVenous Immunoglobulin
JM109 (DE3)	Bacterial *E.coli* strain
kDa	KiloDalton
LB	Luria Bertani Miller Broth
Lys	Lysine
mAbs	Monoclonal Antibodies
MCS	Multiple Cloning Site
MG	Myasthenia Gravis
MiliQ water	Bi-distilled water
MIR	Main Immunogenic Region
mRNA	Messenger RiboNucleic Acid
MuSK	Muscle-Specific tyrosine Kinase
nm	NanoMetres
NMJ	NeuroMuscular Junction
OD	Optical Density
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction

Pro	Proline
RE	Restriction Enzyme
RIA	RadioImmunoAssay
rpm	Rotations Per Minute
RPMI	Roswell Park Memorial Institute medium
SB	Super Broth
SDS	Sodium Dodecyl Sulphate
SOC	Super Optimal Broth with Catabolite repression
SSC	Side Scatter Channel
TBE	Tris-Borate-EDTA
TBS	Tris Buffered Saline
Trp	Tryptophan
Tyr	Tyrosine
Mg	Microgram
µL	Microliter
UV	UltraViolet
Val	Valine
Xaa	Unspecified amino acid

1. Introduction

1.1. Myasthenia Gravis

Myasthenia Gravis (MG) is an autoimmune disease triggered by antibodies directed against proteins involved in signalling at the neuromuscular junction (NMJ), resulting in failure of the neuromuscular transmission, muscle weakness and abnormal fatigability. It is characterized by gradual impairment of daily activities such as sitting up, brush the teeth, rise from a chair, talk, chew, swallow and even breath. The patients' weakness fluctuates every day and tends to get worse with activity and ameliorates with rest (Kaminski, 2009; Meriggioli *et al.*, 2009).

In Europe the incidence ranges from 3 to 30 per million and the prevalence is between 15 and 179 per million inhabitants. The increasing prevalence registered in the past decade is mostly due to improved diagnostic techniques and treatments that increase longevity. This disease occurs in both genders but the incidence is higher in women (Andersen *et al.*, 2014).

1.1.1. Forms of Myasthenia Gravis

Myasthenia Gravis can be classified based on the location of affected muscles, antibody profile and age of onset (Berrih-Aknin *et al.*, 2014; Kaminski, 2009).

a) Location of affected muscles

The ocular form of MG is diagnosed when symptoms are limited to the ocular muscles for at least two years. The most common symptoms are ptosis, which is the abnormal lowering or drooping of the upper eyelid caused by muscle weakness or paralysis, and/or diplopia, a visual defect in which a single object is seen in duplicate (Meriggioli *et al.*, 2009).

In approximately 15% of patients with MG the symptoms remain limited to the extrinsic ocular muscles, with a greater prevalence in men over the age of 40. However, symptoms may progress to the bulbar muscles (muscles of the mouth and throat responsible for speech and swallowing) and limb muscles, resulting in a generalized form of MG (Berrih-Aknin *et al.*, 2014; Conti-Fine *et al.*, 2006). In these cases the symptoms include dysphagia, dyspnea, dysarthria (difficulty in articulating words caused by impairment of the muscles used in speech) and facial weakness.

When respiratory muscle weakness is acute and severe (myasthenic crisis), tracheal intubation may be necessary for ventilator support or airway protection.

b) Antibody profile

Most of the autoantibodies in patients with Myasthenia Gravis are directed to the nicotinic acetylcholine receptor (≈85%) and are called anti-AchR antibodies (Conti-Fine *et al.*, 2006; Jayam Trouth *et al.*, 2012; Meriggioli *et al.*, 2009; Rodgaard *et al.*, 1987; Sine, 2012; Vincent *et al.*, 1982; Vincent *et al.*, 1980). However other proteins present in the neuromuscular junctions, such as Muscle-Specific Kinase (MuSK) (Conti-Fine *et al.*, 2006; Evoli *et al.*, 2008; Illa *et al.*, 2008; Meriggioli *et al.*, 2009; Zisimopoulou *et al.*, 2013), titin (Yamamoto *et al.*, 2001) and rapsyn (Agius *et al.*, 1998) may also be targeted, resulting in myasthenic symptoms.

Like AchR, MuSK is a postsynaptic transmembrane protein present in muscle cells at the NMJ, and is involved in neuromuscular transmission. In mature neuromuscular synapses, the MuSK protein is part of the agrin receptor (agrin is a protein produced by motor neurons and secreted into the synaptic basal lamina).

In clinical practice, the antibody profile of patients with Myasthenia Gravis is performed using a commercial kit in which AchR is used as antigen (kit Acetylcholine Receptor Autoantibodies (ARAb) RRA KIPIB21021 – DIAsource ImmunoAssays). Patients who present autoantibodies against the AchR (>0,4nmol/L according to the kit's instructions) are designated seropositive, whilst the remaining patients are classified as seronegative patients.

c) Age of onset

Myasthenia Gravis with an onset before the age of 50 is classified as early-onset (EO-MG). Most of these patients have elevated titters of autoantibodies against the acetylcholine receptor. It is reported that there is a female predominance (60-70%) (Berrih-Aknin *et al.*, 2013).

The late-onset form of Myasthenia Gravis (LO-MG) designates the form of disease present in patients whose onset of symptoms occurred between the ages of 50 and 60 years. These patients have autoantibodies that recognize striated muscle proteins other than the AchR and MuSK (double seronegative patients). It is clinically important to distinguish the early-onset MG from the late-onset

MG. This last group is more prone to develop thymoma and is frequently associated with the generalized form of MG with severe respiratory crises.

The very late-onset MG (VLO-MG) is considered when symptoms appear after 60 years of age and there is a male predominance in this form of MG.

1.2. Anti-AchR antibodies in Myasthenia Gravis

Myasthenia Gravis is an organ-specific autoimmune disease mediated by antibodies binding to the post-synaptic acetylcholine receptors. These antibodies found in patients with MG are polyclonal and heterogeneous in their action, as they may interact with distinct areas of the acetylcholine receptors (Meriggioli *et al.*, 2009; Vincent *et al.*, 1982; Vincent *et al.*, 1980; Zisimopoulou *et al.*, 2013). The identification of which classes and subclasses of antibodies are involved in the immune response against the AchR and subsequent myasthenic symptoms may be important, since it determines the underlying pathologic mechanism, (e.g. complement activation and cell cytotoxicity depends on the presence of specific antibodies) (Rodgaard *et al.*, 1987).

Several studies have shown that, in Myasthenia Gravis, anti-AchR antibodies belong to the IgG class with a predominance of the IgG1 and IgG3 subtypes (Meriggioli *et al.*, 2009; Rodgaard *et al.*, 1987; Vincent *et al.*, 1982; Vincent *et al.*, 1980), whilst antibodies against MuSK are mainly IgG4. Several groups have measured these antibodies by different techniques, such as solid-phase radioimmunoassay (RIA) (Shinomiya *et al.*, 1981; Tzartos *et al.*, 1988), ELISA (Papadouli *et al.*, 1993), indirect ELISA (Wang *et al.*, 2010), immunoprecipitation (Hara *et al.*, 1993; Lefvert *et al.*, 1978; Meriggioli *et al.*, 2009) and flow cytometry (Allman *et al.*, 2011) amongst others, (Meriggioli *et al.*, 2009) obtaining similar results.

The serum concentration of anti-AchR IgG antibodies is highly variable amongst patients with the same degree of disease and may be similar between patients with different grades of muscle weakness and so it cannot be directly correlated or predict the severity of the disease. However, if the disease remains stable, the antibody titres have a tendency not to change. Therefore, in an individual patient the antibody concentration in the serum varies with the disease severity: upon treatment the

antibody titres decrease and with increasing severity the concentration of the antibodies rises (Allman *et al.*, 2011; Kaminski *et al.*, 2012; Lefvert *et al.*, 1978; Meriggioli *et al.*, 2009; Rodgaard *et al.*, 1987).

Nevertheless, the correlation between antibody titres and disease severity is not statistically significant, suggesting that the total burden of antibodies present might mask the titres of the specific antibody that is being pathogenic (Willcox *et al.*, 1984), i.e., the antibodies measured may not be the ones directly implicated in the disease. Therefore it is necessary to identify the antibodies that recognize specific immunogenic areas on the receptor or subtypes that might be more pathogenic, and measure them instead of the total amount of antibodies (IgG) (Rodgaard *et al.*, 1987). Rodgaard et al quantified the concentration of IgG1 and IgG3 antibodies from patients with MG, finding that both immunoglobulins tend to increase with disease severity, but only the IgG1 subtype can eventually be related with disease progression. Further studies are necessary to confirm if in fact IgG1 antibodies can be correlated with the progression of Myasthenia Gravis and if they can be used as biomarkers for the severity of the disease.

1.2.1. Consequences of the anti-AchR antibody binding to acetylcholine receptor

The binding of an antibody to the acetylcholine receptor can cause disruption of the neuromuscular junction, with loss of functional AchRs and consequent failure of the neuromuscular transmission by three different pathogenic mechanisms (Conti-Fine *et al.*, 2006; Meriggioli *et al.*, 2009; Tuzun *et al.*, 2013) (Figure 1):

a) Complement activation at the neuromuscular junction – results from the morphological deformation of the post-synaptic membrane and loss of AchR that consequently leads to the impairment of the neuromuscular transmission. This mechanism also induces a decrease of the voltage-gated Na^+ channels, enhancing the action potential threshold of the muscle membrane. Due to specific characteristics of IgG1 and IgG3 immunoglobulins the lysis of the muscle endplate caused by complement activation is consistent with the idea that these subtypes of antibodies are found in patients with MG and might be associated with the onset and progression of the disease.

b) Antigenic modulation – some antibodies against the AchR are capable of linking two molecules hence inducing an accelerated internalization and subsequent degradation of those cross-linked receptors (a process designated as antigenic modulation). The fast endocytosis of the acetylcholine receptors needs to be balanced with an increased synthesis, otherwise there will be a significant loss of AchR availability at the neuromuscular junction leading to muscle weakness and consequently, to the symptoms present in MG. Antigenic modulation is not triggered by all anti-AchR antibodies as the antibodies' capacity to cross-link two molecules is dependent on the localization of the epitope in the receptor.

c) Functional AchR block – in this unusual mechanism the antibodies have affinity to the acetylcholine binding site on the receptor, blocking the access of acetylcholine to that site. Consequently, neuromuscular transmission is interrupted and muscle activation does not occur. Even a low concentration of these anti-AchR antibodies, may be enough to block the access of acetylcholine to the receptor.

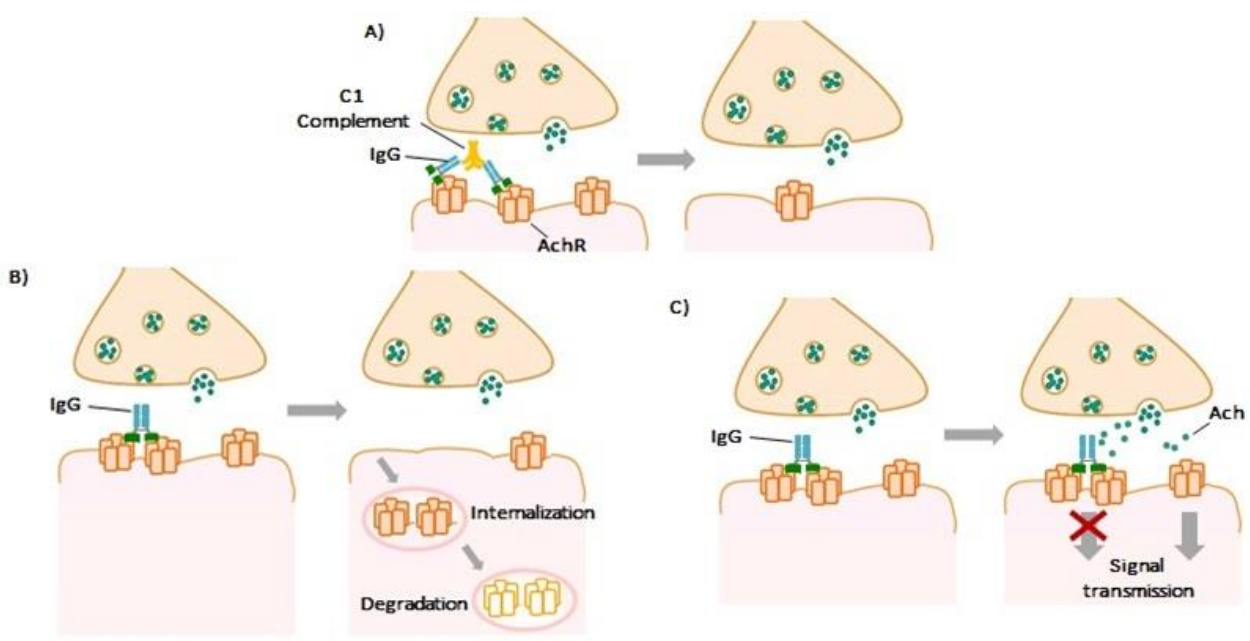

Figure 1 – Consequences of anti-AchR antibody binding to AchR

A) Complement activation at the NMJ with the consequent morphological deformation of the post-synaptic membrane and loss of AchR. B) Antigenic modulation: antibodies against AchR are capable of linking two molecules inducing accelerated internalization and subsequent degradation of those cross-linked receptors. C) Functional AchR block mechanism in which antibodies have affinity to the acetylcholine binding site on the receptor blocking the access of acetylcholine to that site and interrupting neuromuscular transmission.

In Myasthenia Gravis it is possible to have the three pathogenic mechanisms occurring simultaneously, at the same neuromuscular junction, which lowers the efficiency of many therapeutic approaches.

1.3. Treatment of Myasthenia Gravis

The therapeutic approach of MG is based on non-antigen specific immunotherapies. These therapies can be classified into conventional, which includes corticosteroids and immunosuppressive drugs such as azathioprine, mycophenolate mofetil and cyclosporine, or immunomodulating therapies like intravenous immunoglobulin (IVIg) or plasmapheresis. Conventional drugs have demonstrated to be advantageous in the chronic treatment of Myasthenia Gravis, however these drugs are non-specific and their efficiency and tolerance is different between patients. Furthermore, the last group provides only a short-term effect instead of a continuous immunosuppression and therefore is only useful for myasthenic crisis (Collongues *et al.*, 2012; Dalakas, 2012; Dalakas, 2013; Dalakas, 2006; Gold *et al.*, 2003; Kaminski, 2009; Meriggioli *et al.*, 2008; Wylam *et al.*, 2003).

1.3.1. New therapeutic drug development for Myasthenia Gravis

Despite the progress and quality improvement of the conventional and immunomodulating therapies there are still some patients that do not respond so well to the available treatments. Considering this, there is an urgent need to explore and develop new drugs in order to treat MG patients with higher specificity and efficiency, long-term effect and with less severe and extensive side effects.

Currently there are several ongoing investigations that attempt to develop new therapeutic drugs for Myasthenia Gravis (Coles *et al.*, 1999; Dalakas, 2008; Dalakas, 2012; Dalakas, 2013; Gold *et al.*, 2003; Weinblatt *et al.*, 1999; Wylam *et al.*, 2003):

- ✓ Monoclonal antibodies (mAbs) consist of a protein constituted by a human or humanized IgG molecule, or in which the hypervariable regions may be from murine origin (chimeric antibody). Some of the monoclonal antibodies available today are used in the treatment of autoimmune diseases like Multiple Sclerosis.
- ✓ Therapeutic fusion proteins are proteins formed by an Fc region of an IgG molecule combined with the extracellular domain of another molecule (e.g. a receptor). These proteins can bind

to antibodies that recognize the molecule and subsequently induce their degradation. Approved fusion proteins are currently used in several diseases: Wet Macular Degeneration, Cryopyrin-Associated Periodic Syndromes (CAPS) and Rheumatoid Arthritis.

There are several drugs that target B-cells in a specific way, (either monoclonal antibodies or fusion proteins), currently being tested in clinical trials. Two of these (Rituximab and Ofatumumab) have been approved by the FDA and are currently used to treat patients with Myasthenia Gravis and Rheumatoid Arthritis amongst other diseases. Both drugs target the CD20 molecule, a membrane-associated phosphoprotein present in the surface of most B-cell, with the exception of stem cells, pro-B-cells and effector B-cells, resulting in the depletion of the circulating B-cells. While Rituximab is directed to a specific region of the CD20 large loop, Ofatumumab recognizes different epitopes: both large and small loops of CD20, which leads to a more effective B-cell lysis (Coles *et al.*, 1999; Dalakas, 2008; Dalakas, 2012; Dalakas, 2013).

Although drug development is essential, it is still important to considerer that these new approaches are very expensive and have long-term safety issues and therefore, the overall balance risk/benefit needs to be taken into account before approving any new therapeutic drug. In Myasthenia Gravis, drug development and the identification of reliable biomarkers are still in the very beginning.

1.4. Acetylcholine receptor

The nicotinic acetylcholine receptor (AchR) is a ligand-gated ion-channel (ionotropic receptor) that mediates the communication between cells (intercellular) in non-neuronal tissues such as bronchial and vascular epithelia and skin, and is extremely important in the signal transmission in autonomic ganglia, in neuromuscular junctions – synapses between motor neurons and skeletal muscle cells – and in the central nervous system, acting both in presynaptic and postsynaptic membranes.

These receptors are formed by five out of 17 known subunits: 10 alpha (α1-α10) subunits, 4 beta subunits (β1-β4), one gamma (γ), one delta (δ) and one epsilon (ε). In contrast to what happens in neuronal subtypes of the receptor, there are only two subtypes of muscle AchR that allow the contraction of muscle cells (Lodish *et al.*, 2000; Quintas *et al.*, 2008) (Figure 2-A).

Considering the clinical characteristics of Myasthenia Gravis, we focussed our attention in the AchRs present exclusively in the NMJ. Each striated muscle nicotinic AchR is formed by two α1, and three non-alpha: β1, δ and one γ or ε subunits, resulting in a pentameric transmembrane protein with one of the following compositions $(\alpha1)_2\beta1\delta\gamma$ or $(\alpha1)_2\beta1\delta\varepsilon$ and a molecular weight of ≈290 kDa (Albuquerque *et al.*, 2009; Conti-Fine *et al.*, 2006; Lodish *et al.*, 2000; Psaridi-Linardaki *et al.*, 2002; Quintas *et al.*, 2008) (Figure 2-B). Acetylcholine receptors with the stoichiometry of $(\alpha1)_2\beta1\delta\gamma$ are mostly found in embryonic stages whilst receptors with the ε subunit ($(\alpha1)_2\beta1\delta\varepsilon$) are found in mature neuromuscular synapses. During the formation of the muscle endplate, the transcription of α1, β1, δ and γ subunits occurs, to form the final receptor ($(\alpha1)_2\beta1\delta\gamma$). The AchR activation and the increasing of cell depolarization, caused by the interaction with its agonists, triggers the transcription of the ε subunit, i.e., the contact of the AchR with acetylcholine up-regulates the expression of the ε subunit. At this point occurs the transcription of both γ and ε subunits which will compete to form the final receptor ($(\alpha1)_2\beta1\delta\gamma$ or $(\alpha1)_2\beta1\delta\varepsilon$). Receptors with the subunit composition $(\alpha1)_2\beta1\delta\varepsilon$ are more stable and have less tendency to be degraded. Additionally these receptors provide a faster response to the ligand activation than the $(\alpha1)_2\beta1\delta\gamma$. Still, there are some muscles that maintain a predominant expression of the γ subunit on acetylcholine receptors such as the extrinsic ocular muscles (Albuquerque *et al.*, 2009; Wanamaker *et al.*, 2003).

The five subunits exhibit a considerable homology in both sequence and structure. All the AchR subunits have a major extracellular domain, with a conserved area of approximately 200 amino acids (aa) in the amino-terminal, which includes a signal peptide of 20 aa, four transmembrane domains (with about 20 amino acids each), and a cytoplasmic loop of variable length and amino acid sequence between the third and fourth transmembrane domains (Figure 2-C). The first three transmembrane domains are extremely conserved and the fourth transmembrane domain has a 10-amino acid extracellular carboxyl-terminal sequence. In the major extracellular domain, all five subunits possess a cysteine-loop (Cys-loop) consisting of two cysteine residues separated by thirteen highly conserved amino acids, linked together by a disulphide bond (Albuquerque *et al.*, 2009; Ching *et al.*, 2011; Lodish *et al.*, 2000; Quintas *et al.*, 2008). The receptor's subunits are designated α and non-α based on the presence or absence

of a cysteine pair (Cys-Cys pair) close to the entry to the first transmembrane domain (residues 192 and 193 of the extracellular domain), which is necessary for the binding of the neurotransmitters (Figure 2-C).

The acetylcholine binds to the receptor at the interface between an alpha subunit and a non-alpha subunit in the extracellular domain of the AchR, i.e. binds at the interface of the αδ and αγ (or αε) subunits (Figure 2-B)(Albuquerque *et al.*, 2009; Cooper *et al.*, 2004; Lodish *et al.*, 2000). The attachment of two acetylcholine molecules to their binding sites on the ionotropic receptor leads to the conformational changes that open the ion-channel allowing Na^+ and K^+ ions to pass across the membrane modifying its potential and resulting in the contraction of the muscle cell. When the acetylcholine binds to the receptor, it causes a rotational force making the second transmembrane domain of each subunit (that form the central pore of the ion-channel) to rotate around a vertical line central to the channel. This results in the replacement of the hydrophobic residues that face the central pore by more hydrophilic residues allowing the ions to flow across the channel (Figure 2-D) (Albuquerque *et al.*, 2009; Conti-Fine *et al.*, 2006; Cooper *et al.*, 2004; Lodish *et al.*, 2000; Quintas *et al.*, 2008).

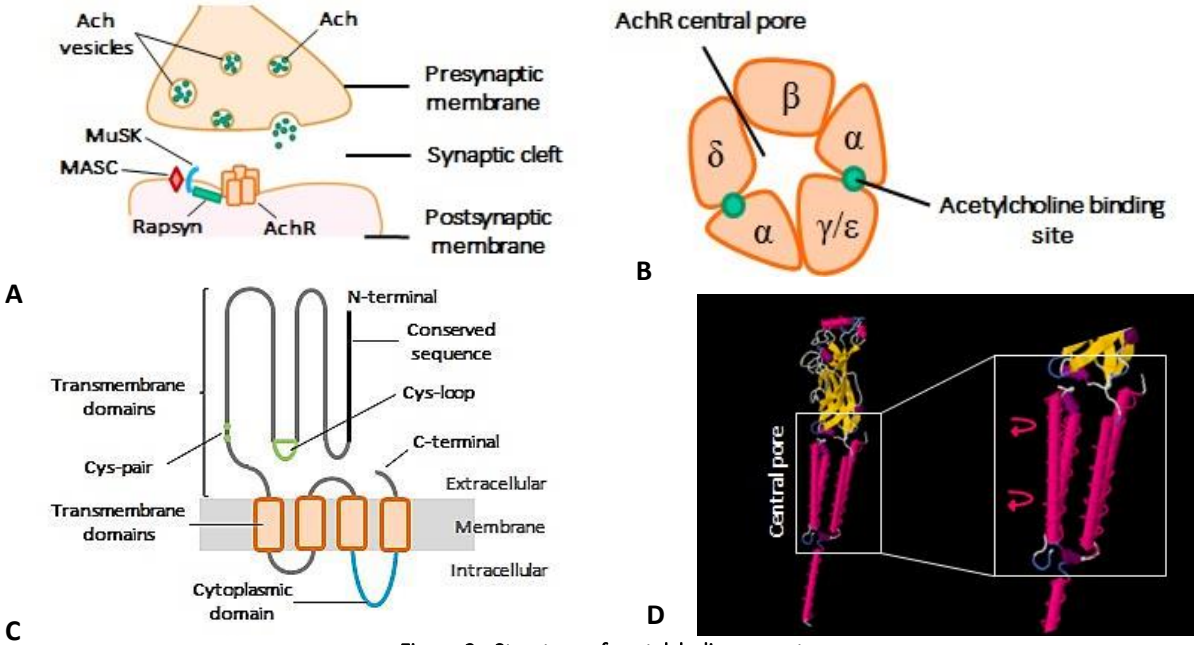

Figure 2 - Structure of acetylcholine receptor.

A) Structure of a NMJ with the AchR and MuSK proteins; **B)** AchR observed from the extracellular space with the identification of each subunit that constitutes the receptor and the two acetylcholine binding sites at the interfaces between the α and γ (or ε) subunits and the α and δ subunits; **C)** Schematic view of the structure common to all AchR subunits: amino-terminal with the conserved 200 amino acids, major extracellular domain with the Cys-loop, four transmembrane domains and cytoplasmic domain between the third and fourth transmembrane domains. It is also represented the Cys-pair found

exclusively on alpha subunits of AchR, crucial for neurotransmitters binding; **D)** Conformational changes caused by the attachment of two acetylcholine molecules to their binding sites on the ionotropic AchR: rotational forces oblige the second transmembrane domain of each subunit to rotate relative to the vertical central line of the channel, leading to the replacement of the hydrophobic residues that face the central pore by more hydrophilic residues allowing the ions to flow across the channel.

1.4.1. Alpha1 subunit of the acetylcholine receptor

In the AchR, the alpha1 subunit is the one implicated in the acetylcholine binding and is the subunit most biologically active. This is why we have focussed our attention in this subunit.

The human alpha1 subunit of the AchR is codified by the CHRNA1 gene located on the chromosome 2 (2q31.1) and initially it was thought that it was divided into eight introns and nine exons (termed P1 to P9), however an additional 75bp coding sequence (corresponding to another exon) located between exons P3 and P4 was described: the exon P3A. So this gene has two isoforms generated through alternate splicing of the primary RNA transcript, that can originate mRNAs with (P3A+ isoform) or without (P3A- isoform) the 25-amino acid sequence of the exon P3A. Both isoforms are present in the human skeletal muscle, nevertheless the P3A+ isoform is not expressed in some animals such as mouse, calf and monkey (Beeson *et al.*, 1990; Morris *et al.*, 1991; Saini *et al.*, 2005), which are some of the experimental models used for Myasthenia Gravis (EAMG).

The isoform that contains the P3A exon results in a 482-amino acid long protein (Figure 3) that includes the acetylcholine binding site and the MIR peptide (see Main immunogenic region) and has a molecular weight of approximately 54kDa (Psaridi-Linardaki *et al.*, 2002). This will be the object of interest of this project.

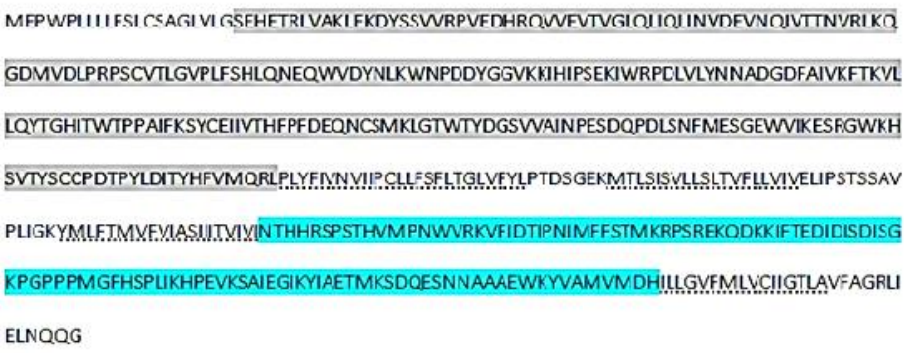

Figure 3 – Amino acid sequence of the P3A+ isoform of the alpha1 subunit of acetylcholine receptor

In grey is represented the sequence corresponding to the extracellular domain of the alpha1 subunit; underlined are represented the four transmembrane domains and in blue is the cytoplasmic domain of the protein.

1.4.2. Main immunogenic region

In MG, most autoantibodies directed to the acetylcholine receptor bind to the extracellular domain of the receptor. It was described (Papadouli *et al.*, 1993; Protti *et al.*, 1990; Tzartos *et al.*, 1991; Tzartos *et al.*, 1988; Tzartos *et al.*, 1992) in animal models of this disease, that there is a small area of the alpha1 subunit, designated main immunogenic region (MIR), distinct from the acetylcholine binding site, that appears to be the main target for antibody binding (Papadouli *et al.*, 1993; Protti *et al.*, 1990; Tzartos *et al.*, 1991; Tzartos *et al.*, 1988; Tzartos *et al.*, 1992). The confirmation of the MIR localization and structure as well as the anti-AchR antibodies involved are essential to design new strategies for the diagnosis and treatment of Myasthenia Gravis.

1.4.2.1. Localization and structure of the main immunogenic region in AchR

Previous studies demonstrated that the MIR is restricted to a 10-amino acid sequence (residues 67-76 of the alpha1 subunit). In them, small peptides equivalent to the alpha1 subunit of AchR, with a length of 14 to 20 residues overlapping each other by 4 to 6 residues, were synthesized, and the attachment of a vast panel of anti-AchR monoclonal antibodies to those peptides was evaluated (Papadouli *et al.*, 1993; Tzartos *et al.*, 1991; Tzartos *et al.*, 1988).

Tzartos et al (Tzartos *et al.*, 1988) measured the binding of fifteen monoclonal antibodies. The majority (eleven monoclonal antibodies) bound strongly to the peptide YNLKWNPDDYGGVKKIHI (of which six bound exclusively to that peptide, two also bound significantly to IEGIKYIAETMKSDQESN – because these monoclonal antibodies recognized the sequences Gly-Ile-Lys-Xaa-Ile or Gly-Val-Lys-Xaa-Ile that are present in both peptides, and three antibodies bound weakly to other peptides). Four monoclonal antibodies did not recognize any peptide, however one must bear in mind that antibody binding might also be dependent on the peptide conformation. Taking into account these findings, these authors created six new peptides that matched distinct regions of the peptide that proved to be the one with more monoclonal antibodies binding to: YNLKWNPDDYGGVKKIHI. This study showed that every monoclonal antibody investigated in this experiment (eight anti-MIR antibodies) linked exclusively to the peptide WNPDDYGGVKWNPDDYGGVK, that includes the decapeptide WNPDDYGGVK which corresponds to the residues 67-76 of the alpha1 subunit (YNLK**WNPDDYGGVK**KIHI) (Tzartos *et al.*, 1991;

Tzartos *et al.,* 1988), suggesting that this peptide has immunodominant properties in the Experimental Autoimmune Myasthenia Gravis (EAMG) model and is the main site of antibody binding in the acetylcholine receptor.

Years later, Papadouli et al (Papadouli *et al.,* 1993) examined the importance of each amino acid residue in the MIR decapeptide by doing several amino acid substitutions with analogous amino acids, and subsequently analysing the binding of monoclonal antibodies to those peptides. They conclude that:

- → The binding of the tested mAbs appears not to be dependent on the Trp67 residue;
- → The residues Asn68, Pro69and Asp71 are essential to the MIR antigenic proprieties, as the substitutions of these amino acids reduce the ability of the mAbs to bind to the peptide;
- → Antibody binding is slightly affected due to substitutions on Tyr72: in a few situations there is an enhancement of the antibody binding to the peptide;

It should be considered that a single amino acid substitution in the MIR peptide can lead to a significant conformational change that may impair the antibody binding and therefore, the results obtained in this study should be considered with caution.

Taking into account that in some residue substitutions there was an intensification of the antibody binding, this group suggested that a new method for the treatment of MG could involve the presence of a synthetic MIR peptide with greater affinity to anti-AchR antibodies, which would prevent the binding of the antibodies to the AchR at the NMJ (Papadouli *et al.,* 1993).

All the studies mentioned above reached their conclusions by using mAbs isolated from mice immunized with the naïve acetylcholine receptor from different species or subunits of AchR purified from *Torpedo Californica* (a specie of electric ray from the *Torpedinidae* family known as *Pacific electric ray)*. This is a wealthy source of muscle-type AchR used as starting material for receptor purification, since the electric organ of these rays contains approximately 40% of this protein (Albuquerque *et al.,* 2009; Kaminski *et al.,* 2012). This can be misleading and can compromise the transition from animal studies to human studies for several reasons: first the immune responses developed by mice differ from the human ones as the characteristics of the antibodies are different between species.

Secondly, the affinity of the antibodies to isolated subunits of the AchR or the MIR synthetic peptides may be distinct from its affinity to the naïve acetylcholine receptor. Additionally, immune responses induced against an outside peptide do not have the same features as autoimmune responses. These issues are crucial when it comes to the validation of the experimental results, since they can lead to the use of antibodies that do not disclose the same type of response or the same binding site on the isolated acetylcholine receptor as those developed by patients with MG.

One possible way to avoid these problems is identifying the B lymphocytes responsible for the production of the antibodies against the peptide of interest (residues 67-76 of alpha1 subunit). Allman et al (Allman *et al.*, 2011) characterized B lymphocytes from peripheral blood of immunized mouse models with AchR purified from *Torpedo Californica* to induce an experimental autoimmune model of Myasthenia Gravis (EAMG). Then, AchRs also purified from *Torpedo Californica* were conjugated with Alexa fluor647 (originating the complex AchR-Alexa fluor647) and used as a probe to identify B lymphocytes that expressed at its surface immunoglobulins with affinity to the receptor, i.e., detect which cells were responsible for the production of autoantibodies against the AchR in EAMG models. Flow cytometry revealed that in EAMG mice the AchR-Alexa fluor647 complex bound to lymphocytes that expressed the glycoprotein B220 at their surface.

B220 is a major transmembranar glycoprotein present in the surface of B lymphocytes, with the molecular weight of 220kDa. In mice, this isoform of the CD45 family is found in initial precursors of B cells and is maintained till the stage of mature B-cells, even after antigen recognition, and is used as a B-cell marker. In humans, B220 expression cannot be used has a marker since its expression is correlated with the CD27 expression, which is a memory B-cell marker. Human B lymphocytes can be divided into three main groups: B220-/CD27- (since the B220 protein is not expressed in all B lymphocytes), B220+/CD27- and B220-/CD27+ (Bleesing *et al.*, 2003; Rodig *et al.*, 2005). Initially it was thought that B220 was found only in naïve B-cells and that this glycoprotein could be used has a marker for human naïve B-cells. CD27 is a marker for memory B-cells and it was hypothesized that the contact of B lymphocytes with an antigen and its activation led to a B220 downregulation and an increase in the

CD27 expression at the cell surface, justifying the presence of lymphocytes B220+/CD27- (naïve B-cells) and B220-/CD27+ (memory B-cells). However, the identification of a small B220+/CD27+ B lymphocyte group suggested that the transition from naïve B-cells to memory B-cells would implicate an in-between group that would express both proteins.

In the same set of experiments the lymphocytes that expressed B220 (B220+ lymphocytes) were characterized into class and subclass, showing that both groups of mice (EAMG and control) expressed IgM antibodies although the titres in EAMG mice were higher. Furthermore, immunoglobulins from the IgG2 subclass were exclusively found in the EAMG group, suggesting that these antibodies are specific for MG in this model (Allman *et al.*, 2011). It is important to notice that IgG2 antibodies found in mice are equivalent to the IgG1 subtype in humans and so, this finding supports previous studies reporting that IgG1 antibodies are present in patients with MG.

Additionally, this group found that B lymphocytes expressing IgG2 immunoglobulins could be correlated with disease severity in mice: the increase of B-cell titres in the peripheral blood was associated with an increase in muscle weakness and more severe disease stages, and therefore the authors proposed that these B lymphocytes could be used as biomarkers for disease severity(Allman *et al.*, 2011). However monoclonal antibodies that recognize B220+cells in mice do not bind to human cells (Rodig *et al.*, 2005) so this proposal might not be applicable to humans.

Furthermore, this study may also have some bias: the paratopes that exist in mice antibodies are different from those in humans, the subtype of antibodies responsible for certain immunogenic pathways vary between species, and the markers present at the surface of B-cells differ in human and mice since in mice the glycoprotein B220 is used as a B-cell marker whilst in humans the presence of this protein depends on the expression of CD27, which in turn does not exist in mice.

1.4.2.2. Localization and structure of the main immunogenic region in human AchR

Studies addressing B lymphocyte characterization have not been done in humans yet and it is therefore very important to use human molecules to validate previous research findings.

The first step required for the transition from animal studies to human research is to place the sequence that corresponds to the MIR peptide identified in previous animal studies (Papadouli *et al.*, 1993; Tzartos *et al.*, 1991; Tzartos *et al.*, 1988) in a human acetylcholine receptor. The use of *in silico* tools allowed the visualization of its structure and possible antigenic characteristics as well as the speculation on what the binding sites for the autoantibodies might be.

As shown in Figure 4 in silico analysis showed that in the human's AchR, the immunodominant region (MIR peptide) is found in a β-sheet of the extracellular domain of the alpha1 subunit between the residues 67-76, which corresponds to the amino acid sequence WNPDDYGGVK, as previously stated.

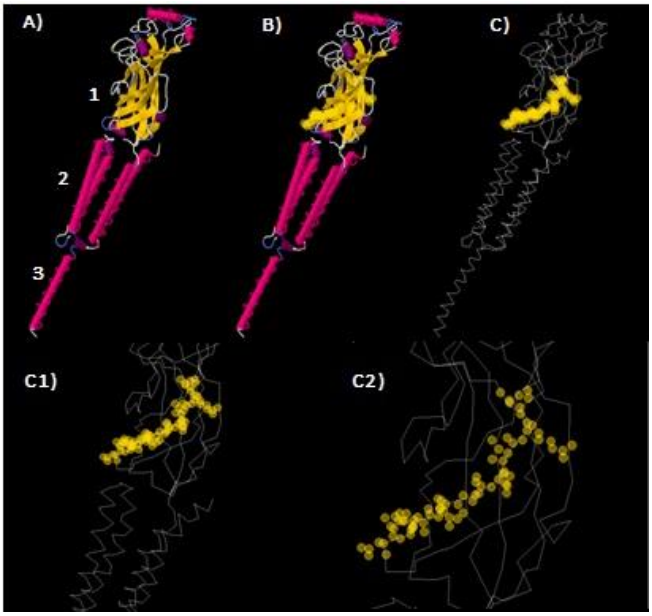

Figure 4 - Structure of alpha1 subunit of the AchR and the MIR localization.

A) Structure of alpha1 subunit: in pink are represented α-helices, in yellow β-sheets and turns are in blue. It is possible to see the large extracellular domain formed by β-sheets and the amino-terminal with the α-helix (1), four transmembrane domains (2) and cytoplasmic domain between the third and fourth transmembrane domains (3); **B)** Structure of alpha1 subunit with the MIR peptide localization(yellow halos) in a β-sheet of the extracellular domain from residues 67-76 (WNPDDYGGVK); **C)** Similar to B), but with a clear view of MIR peptide; **C1)** and **C2)** Amplified views of MIR location from residues 67-76 (WNPDDYGGVK), on the extracellular domain of the alpha1 subunit of AchR.

The next step of these studies would then be to confirm if patients with MG produce autoantibodies that recognize the MIR peptide, as well as, identify the class and subclasses of antibodies produced by these patients. If so, a MIR-like peptide could be used to characterize the B lymphocytes responsible for the production of the pathogenic autoantibodies.

2. Aims

2.1. Overall goal and specific aims

The main target and the antibody profile of patients with Myasthenia Gravis are not yet well-defined. Furthermore not all patients show clinical improvement when treated with the current therapeutic approaches.

This project's overall goal is to investigate the existence of different antigen regions in the nicotinic acetylcholine receptor targeted by autoantibodies produced by patients with Myasthenia Gravis, enabling the use of those targets and/or the corresponding anti-AchR antibodies as biomarkers for both diagnostic and/or treatment response in Myasthenia Gravis.

Considering that one study performed in mice identified one peptide (MIR) present in the alpha1 subunit of the AchR as the main target of antibody binding, it is necessary to determine if patients with Myasthenia Gravis produce autoantibodies against the same MIR peptide (AchR+ and MIR peptide+) and, if so, distinguish them from those that do not recognize the MIR peptide, but do have autoantibodies against other regions of the alpha subunit of the AchR (AchR+ and MIR peptide-). Furthermore, it would be important to identify the B lymphocyte population responsible for the production of the autoantibodies against the AchR, particularly whether they express CD20 as that could provide grounds for the use of specific drugs.

To do so, specific aims were outlined:

a. Develop a fusion protein of the extracellular domain of the alpha 1 subunit of the AchR.
b. Identify whether the MIR peptide and/or other peptides obtained from the production of the extracellular domain of the alpha1 subunit of the AchR are targeted by human antibodies present in serum from patients with MG.
c. Identify and characterize the B lymphocyte sub-population responsible for the production of the autoantibodies in patients with Myasthenia Gravis.

3. Methods

All assays and laboratory procedures described in this chapter were optimized and systematically revised in order to minimize inter and intra-assay variability.

3.1. Patients & Healthy controls

14 patients with Myasthenia Gravis attending Prof. Doutor Fernando Fonseca Hospital, Amadora, Portugal were enrolled in this study, and 14 volunteer healthy subjects sex- and age-matched were also included and used as controls. The current investigation was approved by the Ethics Committees of Prof. Doutor Fernando Fonseca Hospital and all subjects gave informed consent before entering the study.

Blood samples from patients with Myasthenia Gravis and healthy subjects were collected and both serum and peripheral blood mononuclear cells (see procedure below) were isolated and stored at -80ºC until analysis.

3.1.1. Antibody profile

The antibody profile of the patients with Myasthenia Gravis was performed using a commercial kit in which the AchR was used as antigen (kit Acetylcholine Receptor Autoantibodies (ARAb) RRA KIPIB21021 – DIAsource ImmunoAssays). Patients whose serum presented autoantibodies against the AchR (>0,4nmol/L according to the kit's instructions) were designated seropositive, and the remaining patients were classified as seronegative.

3.1.2. Peripheral Blood Mononuclear Cells isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood samples collected in EDTA tubes of both healthy subjects and patients with Myasthenia Gravis by Ficoll-Paque™ density-gradient sedimentation, as described: Ficoll-Paque™ Plus reagent was placed into a 50mL conical tube and carefully overlaid with 20mL of diluted blood sample (dilution 1:1 in PBS containing 1%FBS) with special attention not to mix the two layers. After a centrifugation at 2000rpm for 20 minutes at 20ºC in a swinging-bucket rotor without brake, the mononuclear cell layer (interface layer) was carefully transferred to a new 50mL conical tube, smoothly ressuspend in PBS containing 1% FBS and then centrifuged at 1500rpm for 5 minutes at 4ºC (washing step). The washing step was performed three

times. The samples were then transferred to a non-treated T75 flask (Sigma-Aldrich) and incubated overnight at 37ºC 5%CO_2 in RPMI medium. The PBMCs were counted using a Neubauer Chamber, transferred to a 15mL conical tube and centrifuged one last time at 1500rpm for 5 minutes. The supernatant was carefully removed and the cell pellet was resuspended in a storage reagent (FBS containing 10% DMSO) to a final concentration of [10^6 cells/mL]. All samples were stored at -80ºC until analysis.

3.2. MIR synthetic peptide

Based on the literature review mentioned above (Main immunogenic region), the autoantibodies in EAMG models recognize a specific region of the alpha1 subunit of the AchR designated Main Immunogenic Region with the sequence WNPDDYGGVK. Here, it was intended to confirm if human patients with MG produce autoantibodies that recognize the MIR peptide.

A ten-residue long peptide, designated Main Immunogenic Region (MIR), corresponding to the amino acid residues 67-76 (WNPDDYGGVK) of the extracellular domain of the alpha1 subunit of the human acetylcholine receptor was commercially synthesized (Thermofisher Scientific – VWR), accordingly to what was described in previous studies (Tzartos *et al.*, 1988).

The lyophilized DNA was dissolved in distilled water to a final concentration of 0,9µg/µL. This peptide was assembled containing a fluorescent probe (FICT) and was stored at -80ºC, away from light.

3.2.1. Screening of serum from patients with Myasthenia Gravis for anti-MIR peptide IgG antibodies by an enzyme-linked immunosorbent assay (ELISA)

An ELISA assay was developed to confirm if the serum from patients with Myasthenia Gravis had autoantibodies that would recognize the MIR peptide from the AchR. The ELISA optimization process included testing different coating buffers and peptide concentrations, sample dilutions, blocking and washing buffers and the conjugated antibody as evidenced in Table 1:

ELISA step	Optimization
Plates	**Nunc PolySorp,** 96-well **Nunc Immobilizer,** solid plate 96-well flat bottom

Coating	MIR peptide: 1,5 µg/mL ; 5 µg/mL ; 10 µg/mL Buffer: **sodium carbonate** pH 9,6 / pH 8,3 / pH 6 **ethanol** 70% **Tris-HCl** pH 5 / pH 6 / pH 7,5 Incubation: **overnight** 4ºC ; **2hours** 37ºC
Samples	Dilution: **1:20** / **1:50** / **1:100** / **1:200** PBS 1x containing 1% bovine serum albumin
Blocking buffer	PBS 1x containing **1% bovine serum albumin** PBS 1x containing **3% bovine serum albumin** PBS 1x containing **5% milk**
Washing buffer	**PBS 1x** PBS 1x **containing 0.05% Tween 20**
Antibody Anti-human IgG	Conjugated with: **Alkaline Phosphatase** **Horseradish Peroxidase** Dilution: **1:1000 ; 1:10000** PBS 1x containing 1% bovine serum albumin

Table 1 – ELISA optimization

Conditions and parameters tested to optimize the ELISA protocol.

The titres of anti-MIR IgG antibodies from both patients with Myasthenia Gravis and controls were measured by a final ELISA protocol as follows: Half Nunc 96-well plates (PolySorp) were coated with 1,5 µg/mL synthesized MIR peptide (ThermoFisher scientific – VWR) diluted in ethanol 70% buffer overnight at 4ºC. Subsequently the blocking buffer (PBS 1x containing 3% bovine serum albumin) was added and incubated for 1hour at 37ºC. Plasma samples from patients with Myasthenia Gravis and healthy subjects, diluted 1:100 in PBS 1x containing 1% bovine serum albumin, were added in triplicate to the 96-well plates for 1hour at 37ºC. After washing four times with PBS buffer containing 0.05% Tween 20, an anti-human IgG (Fc specific)-alkaline phosphatase antibody (Sigma-Aldrich) diluted 1:1000 in PBS with 1% bovine serum albumin was added for 1hour. The substrate (4-Nitrophenyl phosphate disodium salt hexahydrate; Sigma-Aldrich) diluted 1:5000 in BIC buffer (pH 9.8) was then added and incubated for 1hour at 37ºC until yielding a yellow colour, and the OD was read in a spectrophotometer at 405 nm with reference to 605 nm (Appendix 1 – ELISA method).

3.2.2. Identification and characterization of the B lymphocyte sub-population with reactivity to the MIR peptide in patients with Myasthenia Gravis by flow cytometry

The acquired PBMCs from both patients with Myasthenia Gravis and healthy controls (see Peripheral Blood Mononuclear Cells isolation**Erro! A origem da referência não foi encontrada.**) were incubated simultaneously (but separately) for 20 minutes at room temperature, away from light with:

a) ‘CD20’ (sample incubated with anti-human CD20 antibody APC-conjugated (Invitrogen));

b) ‘MIR’ (PBMCs incubated with MIR peptide commercially synthesised conjugated with FICT);

c) ‘MIR/CD20’ (double staining);

A negative control (‘Cells’ – PBMCs without staining) was used in every assay.

The protocol optimization was required and several dilutions were tested as evidenced in Table 2:

Staining step	Optimization
Anti-human CD20 antibody APC-conjugated (Invitrogen)	Dilution: 1:500 1:1000 1:5000 PBS 1x
MIR synthetic peptide conjugated with FICT (commercially synthesised)	Dilution: 1:50 1:100 PBS 1x

Table 2 – Flow cytometry optimization

Conditions tested to optimize the flow cytometry protocol

In the optimized final protocol, the dilutions selected to analyse the samples were: anti-human CD20 antibody APC-conjugated (Invitrogen) diluted 1:1000 in PBS1x buffer and the MIR peptide conjugated with FICT diluted 1:50 in PBS1x buffer.

After 20-minute incubation, 800µL of PBS1x buffer were added to each eppendorf (washing step) and the samples were centrifuged at 5000rpm for 2 minutes at room temperature. The supernatant was carefully removed and the cell pellet was resuspended in 400µL of PBS1x buffer.

The samples were analysed in a Flow cytometer (Attune® Acoustic Focusing Cytometer – Applied Biosystems) with *Attune® Cytometric Software*. Cells were gated with a combined live gate, according to the scatter characteristics and the dead cells were excluded.

3.3. Development of a protein composed of the extracellular domain of the alpha 1 subunit of the AchR

The autoantibodies present in patients with MG are biologically active and that the alpha1 subunit is the most functionally important subunit of the AchR, it is likely that the antibody target(s) are in this subunit, therefore in this task we aimed at synthetizing the isolated extracellular domain of the AchR’s

alpha1 subunit (CHRNA1) in order to create a tool for screening the serum of patients with MG for the presence of autoantibodies.

3.3.1. Cloning 1 – CHRNA1 protein

The protein cloning was performed using specific primers and restriction enzymes (RE), in which the sequence corresponding to the extracellular domain of the alpha1 subunit of the human nicotinic acetylcholine receptor (Figure 3) was inserted in a specific region of a cloning vector.

3.3.1.1. Digestion – *pT7* Vector

The digestion protocol followed specific conditions:

- ✓ A total of 20µL of reaction volume was used per 1µg de DNA;
- ✓ 1µL of each enzyme was used per 1µg DNA and the enzyme volume never exceeded 10% of the final reaction volume;
- ✓ The enzyme buffer represented 10% of the final reaction volume.

The plasmid chosen for this task was the *pT7* (Sigma-Aldrich) which allows the production of the protein of interest with a Histidine-TAG. This vector has approximately 5000bp, and contains a gene that confers resistance to ampicillin, a multiple cloning site (MCS) with restriction sites for several enzymes including the *SfiI,* and a Histidine TAG plus HA sequence (Appendix 2 – *pT7 vector*). This vector requires the use of *E. coli* cells containing a source of T7 polymerase, such as BL21 (DE3) cells.

The vector referred above was digested according to the following protocol:

Reagents	Volume
Bi-distilled water	35,2 µL
10x buffer G	6 µL
Vector (*pT7*)	12,8 µL
RE1 (*Sfi I*)	6 µL
Reaction volume	60 µL

Table 3 – *pT7* digestion reaction

The reaction volume was incubated in a thermal cycler at 50ºC overnight. The digestion products were separated on a 1% agarose gel containing 0,01% ethidium bromide. DNA loading dye 6x (Fermentas) was added to the samples (10% of sample volume) and GeneRuler 1kb Plus DNA ladder (Thermo

Scientific) was loaded to one well. The results were visualized and photographed in UV light (ChemiDoc - BioRad).

The products of interest were isolated using the Invisorb® Spin DNA extraction kit (Invitek GmbH, Berlin, Germany) according to the manufacturer instructions: resorting to a UV transilluminator, the DNA band corresponding to the correct weight of the DNA of interest was excised (minimizing the agarose gel slice), placed into a 1,5mL microcentrifuge tube and weighed. Gel Solubilizer S buffer was added to the reaction tube containing the DNA-fragment and incubated at 50ºC for 10 minutes until the agarose gel slice was completely dissolved. After adding Binding Enhancer buffer the mixture was loaded onto the Spin Filter and centrifuged at 11000x g for 2 minutes. The filtrate was discarded and if the reaction volume was >800µL (maximum volume of each spin filter column), the centrifugation step was repeated. Two washing steps were performed (buffer supplied by the kit) and after discarding the flowthrough, a centrifuge step was performed at full speed for 4 minute to remove the residual ethanol of the Wash buffer. The Spin Filter was transferred into a new 1,5mL receiver tube and the DNA sample eluted with 20µL of MiliQ water. The DNA was quantified using NanoDrop and the obtained sample was stored at -20ºC until the DNA/vector binding step.

3.3.1.2. Digestion – CHRNA1 insert

The sequence corresponding to the human extracellular domain of the human alpha1 subunit of the acetylcholine receptor (CHRNA1) (Figure 3) was commercially assembled (LifeTechnologies) from synthetic oligonucleotides and/or PCR products and cloned into a *pMA* vector (plasmid with ≈3000bp) with ampicillin resistance (*ampR*) using *KpnI* and *SacI* cloning sites (Appendix 3 – *Synthetic gene CHRNA1*). The plasmid DNA was purified from transformed bacteria, the concentration determined by UV spectroscopy and the final construct was verified by sequencing. 5 µg of the plasmid preparation were lyophilized for shipping (LifeTechnologies). The lyophilized DNA was dissolved in 50 µL distilled water to a final concentration of 0,1 µg/ µL.

To obtain the DNA of interest (insert) the above referred plasmid was digested according to the following protocol:

Reagents	Volume
Bi-distilled water	12 µL
buffer 10xTango	4 µL
DNA commertially synthesized	20 µL
RE1 (*EcoRI*)	2 µL
RE2 (*NcoI*)	2 µL
Reaction volume	40 µL

Table 4 – CHRNA1 digestion reaction

The reaction volume was incubated in a thermal cycler at 37ºC for 4 hours. The products were separated on a 1% agarose gel and isolated using the Invisorb® Spin DNA extraction kit (Invitek GmbH, Berlin, Germany) according to the manufacturer instructions as mentioned above (see Digestion – *pT7* Vector). The DNA was quantified using NanoDrop and the obtained DNA sample was stored at -20ºC until the DNA/vector binding step.

3.3.1.2.1. CHRNA1 for *pT7* vector

The gene of interest (CHRNA1) digested in the previous step was amplified with specific primers in order to be used in a *pT7* vector. These primers include restriction sites for the *SfiI* enzyme (underlined): 5' ATTGCAGTGGCACTGGCTGGTTTCGCTACCGTGGCCCAGGCGGCCTCCGAACATGAGACCCG TCTG 3' (*alpha-F-Sfi* primer) and 5' CGGGTATGCGCCATGGTGATGGTGATGGTGCTGGCCGGCCTGGCCGT CGGGGC AGCAGGAATA 3' (*alpha-R-Sfi* primer).

The reaction volumes were prepared for DNA amplification accordingly to the following protocol:

Reagents	Positive	negative
Bi-distilled water	20,7 µL	41,5 µL
10x buffer Reaction	5 µL	5 µL
Template DNA (150ng)	20,8 µL	-
Alpha-F-Sfi primer (20pmol/µL)	1 µL	1 µL
Alpha-R-Sfi primer (20pmol/µL)	1 µL	1 µL
dNTPs Mix (0,5mM)	1 µL	1 µL
NZYproof DNA polymerase (2,5U/µL)	0,5 µL	0,5 µL
Reaction volume	50 µL	50 µL

Table 5 – CHRNA1 amplification reaction

The reaction volume was incubated in a thermal cycler with the following PCR reaction conditions:

Cycle step	Temperature	Time	Cycles
Initial denaturation	95ºC	2 minutes	1
Denaturing	95ºC	60 seconds	30
Annealing	58ºC	60 seconds	
Extension	72ºC	50 seconds	

Final extension	72ºC	10 minutes	1

Table 6 – PCR amplification conditions

The products were separated on a 1% agarose gel and the DNA was isolated using the Invisorb® Spin DNA extraction kit (Invitek GmbH, Berlin, Germany) according to the manufacturer instructions as previously mentioned (see Digestion – *pT7* Vector). The DNA was quantified using NanoDrop and the samples were stored at -20ºC until the DNA/vector binding step.

3.3.1.3. CHRNA1-*pT7* binding

Once the vector of interest was opened and the insert was digested, the binding reaction took place. First it was necessary to optimize the DNA/Vector proportion, considering the formula:

$$ng\ of\ insert = \frac{vector\ in\ ng}{[vector\ size\ (\text{bp})/insert\ size\ (\text{bp})]} \times (\text{vector:insert proportion})$$

To bind the CHRNA1 DNA amplified with specific primers to the *pT7* vector the reactions were made resorting to a kit (Gibson Assembly Master Mix Kit - BioLabs) accordingly to the manufacturer instructions, where it was recommended to test the DNA/Vector ratios of 3:1. Using the formula previously presented:

. Ratio 3:1

$$ng\ of\ insert = \frac{40\ ng}{[4197\ \text{bp}/681\ \text{bp}]} \times (3) = 19{,}5\ \text{ng of insert}$$

The reaction volumes were prepared as follows:

Reagents	Reactions	
	Positive (vector + insert) Proportion 3:1	Negative (without insert)
pT7 vector (50ng)	2,9 µL	2,9 µL
Insert (21,42ng)	0,2 µL	-
Kit buffer	10 µL	10 µL
Bi-distilled water	6,9 µL	7,1 µL
Reaction volume	20µL	20µL

Table 7 - CHRNA1 binding to *pT7* vector according to the kit manufacturer instructions

The reactions were incubated in a thermal cycler at 50ºC for 15 minutes. The binding reaction products were transformed into JM109 bacterial cells by electroporation as follows: 2µL of the binding reaction products were transferred to an eppendorf containing JM109 cells, the total volume (cells +

DNA) was resuspended and transferred to the electroporation cuvettes and electroporated at 1,8volt (Gene Pulser II – Bio-Rad). 500µL of SOC medium were added to the cuvette and resuspended. The total volume was transferred to an eppendorf and incubated for 1 hour at 37ºC and 220rpm. The cells were plated in LB agar medium with ampicillin and incubated overnight at 37ºC. The plates were stored at 4ºC until the screening task.

3.3.1.4. Screening by PCR

In the screening protocol for the *pT7*-CHRNA1 binding, several colonies were selected from the ampicillin-LB agar plates and placed in 25µL of sterile water (and stored at 4ºC):

Reagents	positive (to each colony selected)	negative
Bi-distilled water	36,5 µL	41,5 µL
10x buffer Reaction	5 µL	5 µL
Template DNA	5 µL	-
Alpha-F-Sfi primer (20pmol/µL)	1 µL	1 µL
Alpha-R-Sfi primer (20pmol/µL)	1 µL	1 µL
dNTPs Mix (0,5mM)	1 µL	1 µL
NZYproof DNA polymerase (2,5U/µL)	0,5 µL	0,5 µL
Reaction volume	50 µL	50 µL

Table 8 – PCR screening reactions

A PCR similar to the one used in the CHRNA1 DNA amplification was used for the screening step (see CHRNA1 for *pT7* vector section). The samples were separated in a 1% agarose gel following the protocol as described above. The clones were considered positive if, after PCR screening, was possible to see the vector and the insert with the correct molecular weight.

3.3.1.5. CHRNA1 protein expression assay in BL21 bacterial cells

From the clones considered positive in "Screening by PCR", an inoculum was prepared in 7mL of LB medium with ampicillin (dilution 1:1000) and incubated overnight at 37ºC at 220rpm. From each inoculum a stock of each positive cloned bacteria was stored at -80ºC in a 15% glycerol buffer (using 1mL of the inoculum) and the DNA was extracted using 3mL of the inoculum per each clone according to the manufacturer instructions (Miniprep kit – Nzytech). The DNA was quantified in the NanoDrop.

The binding products were transformed in BL21(DE3) bacterial cells by electroporation as follows: 2µL of binding reaction were transferred to an eppendorf containing BL21(DE3) cells, ressuspended and

the total volume (cells + DNA) was loaded to the electroporation cuvettes and electroporation at 1,8volt (Gene Pulser II – Bio-Rad) was performed. 500µL of SOC medium were added to the cuvette and resuspended. The total volume was transferred to an eppendorf and incubated for 1 hour at 37ºC and 220rpm. The cells were plated in LB agar medium with ampicilin and incubated overnight at 37ºC.

One bacterial colony from each positive clone was selected from the plates and placed in separated tubes with approximately 5 mL of SB medium with ampicillin (dilution 1:1000) at 37ºC at 220rpm for 16-18h (plates were stored at 4ºC). An inoculum from each clone was incubated at 37ºC at 220rpm until optimal optical density was achieved (0,8-0,9). Subsequently cell growth was stopped on ice. Protein expression was induced with IPTG 1mM and the cultures were incubated for 3hours at 200rpm at 37ºC. After the protein expression the culture volumes were centrifuged at 4000rpm for 5 minutes and the pellet stored at -20ºC until protein purification.

3.3.1.5.1. Screening of CHRNA1 protein expression

The pellet obtained in the preceding step was resuspended in 400µL of PBS buffer with antiprotease freshly prepared (Roche). The samples were placed in a sonicator for 30 minutes and then centrifuged at 1200rpm for 10 minutes at 4ºC. The soluble fraction was tested in an ELISA assay to assess the protein expression: a polysorp 96-well plate (Nunc) was coated with 100µL of the soluble fraction obtained from each clone, for 1hour at 37ºC. After washing the wells with 200µL of PBS 1x buffer three times, 100µL of blocking buffer (PBS 1x buffer containing 3% albumin) was added to each well and incubated for 1 hour at 37ºC. Anti-HA antibody conjugated with HorseRadish Peroxidase (HRP) was added (dilution 1:1000 in PBS buffer) and incubated for 1hour at 37ºC. Finally ABTS + H_2O_2 (1000:2) was added and the plate was incubated at room temperature until yielding colour (approximately 10 minutes) and the optical density (OD) was read in a spectrophotometer at 405 nm with reference to 492 nm.

In addition to this ELISA expression assay, a western blot was performed to confirm the protein expression. The protocol was equal to the one described in “Screening of fusion protein expression”

but the antibody used in this case was anti-HA antibody conjugated with HorseRadish Peroxidase (HRP).

The clones that expressed the protein of interest were sent for sequencing (at Macrogen). Once the DNA sequence was confirmed to be exactly the same as the one from the original gene of CHNRA1, the protein expression was optimized to be performed in larger amounts.

The expression conditions tested were: 6h of incubation at different temperatures: 16ºC, 20ºC and 30ºC and agitation: 200rpm and 140rpm. After the protein expression, each culture volume was centrifuged at 4000rpm for 5 minutes and the pellet was stored at -20ºC until the protein purification step. Each pellet was resuspended in PBS buffer containing antiprotease (Roche), placed in a pulse sonicator for 20 minutes and centrifuged at 10000rpm for at least 2 hours at 4ºC. The soluble fraction was used to test the protein expression conditions.

The protein expression was verified by electrophoresis: protein samples obtained from bacterial culture from each expression conditions tested were mixed with 4x Protein Loading buffer, denatured for 10 minutes at 95ºC and separated in a 10% SDS-polyacrylamide gel. After running the gel at 20mA for approximately 100 minutes, the system was tuned off, disassembled, the gel was removed (stacking gel was cut off and discarded) and stained with Coomassie Blue for 30 minutes. Distaining buffer (40% distilled water, 10% acetic acid and 50% methanol solution) was added and incubated overnight with agitation.

The Coomassie Blue staining analysis allowed defining the best conditions for protein expression, concluding that the optimized conditions for protein expression of a 2L bacterial culture (in a 5L Erlenmeyer for better ventilation) after IPTG induction, for the selected clone were 6h at 30ºC with agitation at 140rpm.

3.3.1.5.2. Purification of CHRNA1 protein

The CHRNA1 protein produced by cloning was purified by affinity chromatography using HIS-Trap columns (GE Healthcare), since the resulting protein has an 8-histidine fragment at its end.

Briefly, the sample was loaded into the chromatography columns under specific conditions that favour the binding of the protein to the complementary substance present in the column matrix. Changing the conditions (by adding a competitive ligand or altering the pH, ionic strength or polarity) was possible to elute the protein of interest apart from the other proteins.

According to the manufacturer instructions it is recommended to test a gradient of imidazole concentration in protein binding and elution buffers (20mM sodium phosphate, 500mM of sodium chloride, 10 to 500mM of imidazole in 1L; pH7,4 adjusted with hydrochloric acid and filtered through a 0,45µm filter) to optimize the conditions to separate the protein of interest from other proteins existent in the bacterial cultures. In the binding buffer the concentrations of 20mM and 40mM of imidazole were used and for the elution buffer a gradient of imidazole was used: 40mM; 60mM; 100mM; 140mM; 200mM and 500mM. Each fraction passing through the column was collected and separated on a 10%SDS-polyacrimalide gel later stained with Coomassie Blue.

3.3.2. Screening of serum from patients with Myasthenia Gravis for anti-CHRNA1 protein IgG antibodies by an enzyme-linked immunosorbent assay (ELISA)

In this task, the indirect ELISA assays were used to confirm if patients with MG had autoantibodies that recognized the CHRNA1 protein obtained in the "Cloning 1 – CHRNA1 protein" task. After optimization (coating buffers tested: PBS 1x; BIC and ethanol 70% - and protein concentration tested: 10µg/mL and 5µg/mL), the protocol used (similar to the one previously described in Screening of serum from patients with Myasthenia Gravis for anti-MIR peptide IgG antibodies by an enzyme-linked immunosorbent assay (ELISA)) was the following: half Nunc 96-well plates (PolySorp) were coated with 5µg/mL CHRNA1 protein diluted in ethanol 70% overnight at 4ºC. Subsequently, blocking buffer (PBS 1x containing 3% bovine serum albumin) was added and incubated for 1hour at 37ºC. Plasma samples from patients with Myasthenia Gravis and healthy subjects, diluted 1:100 in PBS 1x containing 1% bovine serum albumin, were added to triplicate wells to the 96-well plates for 1hour at 37ºC. After washing four times with PBS buffer containing 0.05% Tween 20, anti-human IgG (Fc specific)-alkaline phosphatase antibody (Sigma-Aldrich) diluted 1:1000 in PBS with 1% bovine serum albumin was added

for 1hour. Substrate (4-Nitrophenyl phosphate disodium salt hexahydrate; Sigma-Aldrich) diluted 1:5000 in BIC buffer (pH 9.8) was added and incubated for 1hour at 37ºC until yielding a yellow colour development and the OD was read in a spectrophotometer at 405 nm with reference to 605 nm.

3.4. Development of a fusion protein composed of the extracellular domain of the alpha 1 subunit of the AchR and the Fc region of a immunoglobulin

This assignment aimed at creating a fusion protein constituted by the extracellular domain of the alpha1 subunit of the AchR (CHRNA1), and the Fc region of an IgG immunoglobulin to create a tool that would allow the identification of autoantibody-producing B-cells in patients.

3.4.1. Cloning 2 – fusion protein

3.4.1.1. Digestion – *pFUSE-hIgG1e1-Fc2* Vector

This digestion protocol followed the specific conditions of the ones described previously for the Digestion – *pT7* Vector.

The plasmid used in this task was the *pFUSE-hIgG1e1-Fc2* (Invivogen) which allows the secretion of proteins fused to an immunoglobulin's Fc-domain. This vector has approximately 4197bp and includes: an IL2 signal sequence (IL2ss) to create Fc-fusion proteins from proteins that are not naturally secreted, the *Sh ble* gene (from *Streptoalloteichus hindustanus*) that confers resistance to Zeocin, and a multiple cloning site (MCS) with the restriction sites for several enzymes including the *EcoRI* and the *NcoI* (Appendix 4 – *pFuse-hIgG1e1-Fc2 vector*).

The above referred plasmid was digested according to the following protocol:

Reagents	Volume
Bi-distilled water	41,5 µL
buffer 10xTango	6 µL
Vector (*pFuse-hIgG1e1-Fc2*)	6,5 µL
RE1 (*EcoRI*)	3 µL
RE2 (*NcoI*)	3 µL
Reaction volume	60 µL

Table 9 - *pFuse-hIgG1e1-Fc2* digestion reaction

The reaction volume was incubated in a thermal cycler at 37ºC for 4 hours. The products were separated on a 1% agarose gel and the DNA was isolated using the Invisorb® Spin DNA extraction kit

(Invitek GmbH, Berlin, Germany) according to the manufacturer instructions and as previously described (see Digestion – pFUSE-hIgG1e1-Fc2 Vector). The DNA was quantified using NanoDrop and the obtained samples were stored at -20ºC until the DNA/vector binding step.

3.4.1.2. CHRNA1 for the *pFUSE-hIgG1e1-Fc2* vector

The sequence corresponding to the human extracellular domain of the alpha1 subunit of the acetylcholine receptor (CHRNA1) was previously digested and separated from its original plasmid in the Digestion – CHRNA1 insert task. In this task the DNA purified from the agarose gel as described before was used. The DNA was quantified using NanoDrop and the obtained sample was stored at -20ºC until the DNA/vector binding step.

3.4.1.3. CHRNA1-*pFUSE-hIgG1e1-Fc2* binding

To optimize the DNA/Vector proportion we considered the following formula:

$$ng\ of\ insert = \frac{vector\ in\ ng}{[vector\ size\ (\mathrm{bp})/insert\ size\ (\mathrm{bp})]} \times (\mathrm{vector{:}insert\ proportion})$$

It was recommended to test the DNA/Vector with ratios of 3:1 and 5:1. Using the formula previously presented:

. Ratio 3:1

$$ng\ of\ insert = \frac{40\ ng}{[4197\ \mathrm{bp}/681\ \mathrm{bp}]} \times (3) = 19{,}5\ \mathrm{ng\ of\ insert}$$

. Ratio 5:1

$$ng\ of\ insert = \frac{40\ ng}{[4197\ \mathrm{bp}/681\ \mathrm{bp}]} \times (5) = 32{,}5\ \mathrm{ng\ of\ insert}$$

The *pFUSE-hIgG1e1-Fc2* – CHRNA1 binding reactions were made as follows:

Reagents	Reactions		
	Positive (vector + insert) Proportion 3:1	Negative (without insert)	Positive (vector + insert) Proportion 5:1
pFUSE vector (40ng)	1,1 µL	1,1 µL	1,1 µL
Insert	2,7 µL	-	4,5 µL
10x buffer R	2 µL	2 µL	2 µL
T4 DNA ligase	0,2 µL	0,2 µL	0,2 µL
Bi-distilled water	14 µL	16,7 µL	12,2 µL
Reaction volume	20µL	20µL	20 µL

Table 10 - *pFUSE-hIgG1e1-Fc2* – CHRNA1 binding reactions

The mixtures were incubated in a thermal cycler at 22ºC for 2 hours plus 5 minutes at 70ºC (to inactivate the T4 DNA ligase enzyme). The reaction tubes were kept at 4ºC until use (maximum 24 hours).

1µL of the DNA extracted from each clone was transformed into JM109 bacterial cells and plated in LB agar plates with the selective antibiotic (Zeocin), using the same protocol as the one described above (CHRNA1-pT7 binding).

3.4.1.3.1. Screening by alkaline lysis

A screening step performed by alkaline lysis was necessary to verify if the bacterial colonies that grew in the Zeocin-LB agar plate had the correct DNA sequence. To perform this protocol it was necessary to prepare three different lysis solutions:

Lysis solution	Reagents	Recipe	Storage
I	10mM EDTA (pH 8) 50mM glucose 25mM Tris-HCl (pH 8)	100mL Tris-HCl: 0,30g Tris + 90mL H_2O, pH adjusted with HCl, final volume completed to 100mL with MiliQ water; The solution was autoclaved; 2mL of 10mM EDTA (pH 8) and 0,99g of glucose were added.	4ºC
II	0,2N NaOH 1% (p/v) SDS	10mL: 0,10g SDS + 400 µL NaOH 5N; final volume completed to 100mL with MiliQ water;	Freshly prepared; Kept at 4ºC
III	5M potassium acetate Acetic acid	100mL: 60mL of 5M potassium acetate, 11, 5mL of acetic acid and 28,5mL of MiliQ water	4ºC

Table 11 – Lysis solutions for alkaline lysis

HCl = hydrochloric acid; SDS = Sodium Dodecyl Sulphate; NaOH = Sodium Hydroxide

Some positive and, at least, one negative bacterial colonies were selected from the plates and placed in separated tubes with approximately 5mL of LB medium with zeocin at 37ºC at 220rpm for 16-18 hours (plates were stored at 4ºC). Afterwards 1,5mL of each inoculum was transferred to an eppendorf and centrifuged at 1300rpm for 5 minutes at 4ºC (the remaining inoculum was stored at 4ºC). The supernatant was discarded and the pellet was resuspended in 100µL of Lysis solution I. 200µL of lysis solution II (freshly prepared) was added and tubes were gently mixed by inversion. After 5-minute incubation on ice, 150µL of lysis solution III were added to each tube and mixed by inversion. The volume was incubated for 5 minutes on ice and then centrifuged at 1300rpm for 5 minutes at 4ºC.

The supernatant was transferred to a new eppendorf and the DNA precipitation was performed with ethanol as follow: 1mL of absolute ethanol was added to each tube and mixed carefully, incubated for 2 minutes at room temperature and centrifuged at 1300rpm for 5 minutes at 4ºC. The supernatant was removed by inverting the tube and 1mL of ethanol 70% was added and mixed by carefully inverting the tube 4 times. After centrifugation at 1300rpm for 2 minutes at 4ºC the supernatant was removed by aspiration, the pellet was dried up at room temperature and resuspended in 40µL of sterile MiliQ water. The DNA was quantified in the NanoDrop and the products were stored at -20ºC.

The DNA isolated from the selected colonies was digested using the same protocol as before (in Digestion – *pFUSE-hIgG1e1-Fc2* Vector):

Reagents	Volume
Bi-distilled water	12 µL
buffer 10xTango	2 µL
Clones' DNA purified by alkaline lysis	5 µL
RE1 (*EcoRI*)	0,5 µL
RE2 (*NcoI*)	0,5 µL
Reaction volume	20 µL

Table 12 – Clones' DNA digestion reactions

The reaction volume was incubated in a thermal cycler at 37ºC for 12 hours. The products were separated on a 1% agarose gel containing 0,01% ethidium bromide. DNA loading dye 6x (Fermentas) was added to the samples (10% of sample volume) and GeneRuler 1kb Plus DNA ladder (Thermo Scientific) was loaded to one well. The results were visualized and photographed in UV light (ChemiDoc - BioRad). The clones were considered positive if, after digestion and separation in the agarose gel, was possible to see the vector and the insert with the correct molecular weight.

3.4.1.4. CHRNA1-Fc fusion protein expression assay by calcium phosphate transfection

When working with mammalian cells special concerns ought to be considered. These cells have a doubling time of 12 to 48 hours being susceptible to contamination by bacteria and fungi therefore specific sterile conditions and procedures were carefully maintained during all assays.

A calcium phosphate transfection protocol was performed to express the protein of interest. In this technique the DNA was endocytosed (by uncharacterized pathways) into HEK-293T cells (Human Embryonic Kidney cell line), following a chemical environment that results in the DNA attachment to

the cell surface (Kingston, 2003). This technique is commonly used with adherent cells to produce stable integrated DNA transfections and tends to be more efficient in the production of fusion proteins in stable cell-lines than other types of transfection such as DEAE-dextran-mediated transfection.

The transfection protocol was performed as described: HEK-293T cells were plated in a 6-well plate at a concentration of 5 x 10^5 cells/well in 2mL of DMEM medium (Sigma-Aldrich) supplemented with 10% Fetal Bovine Serum (FBS) (ThermoScientific) and incubated at 37ºC, in an atmosphere of 95% air and 5% CO_2 at saturating humidity for 16-18 hours.

To perform this protocol it was necessary to prepare two solutions: "Mix A", which contains Tris-Borate-EDTA (TBE) buffer, the DNA of interest (DNA recovered from all positive clones selected in the previous task – Screening by alkaline lysis) and calcium chloride ($CaCl_2$) 2,5M buffer, and "Mix B" that consists in Hepes Buffered Saline (HBS) 2x. These mixes were freshly prepared per each well, using the sterilized stock solutions of TBE 5x buffer (54g Tris-Base; 27,5g of boric acid (H_3BO_3); 20mL EDTA 0,5M; distilled water till 500mL; pH 7,6) and HBS 2x buffer (8g of sodium chloride (NaCl); 0,2g of disodium phosphate heptahydrate (Na_2HPO_4-$7H_2O$); 6,5g HEPES; distilled water till 500mL; pH 7):

Mix	Reagents	Recipe	Sterilization	Storage
A	TBE buffer, 5µg of DNA $CaCl_2$ 2,5M buffer	Per well: TBE buffer up to 250 µL, DNA and 25 µL of $CaCl_2$ 2,5M buffer, following this exact order	Buffers sterilized by filtration with 0,2µm filters	Room temperature
B	HBS 2x	Per well: 250 µL of HBS 2x		Room temperature

Table 13 – Mixes 'A' and 'B' for calcium phosphate transfection

"Mix A" was carefully added to the "Mix B" in continuous agitation (vortex) and incubated for 30 minutes at room temperature. Subsequently the mixes were added dropwise to each well and plates were incubated 24 hours at 37ºC in an atmosphere with 5% CO_2.

24 hours after the transfection the medium was removed from the plates and substituted by DMEM 0 medium (without FBS since it affects protein purification) and incubated for 48 hours at 37ºC in an atmosphere with 5% CO_2. After 48 hours the supernatant was removed and stored at -80ºC until

protein purification. A negative (without DNA) and a positive (DNA known to be expressing a protein with the same molecular weight as the fusion protein tested) control were included in every step.

3.4.1.4.1. Screening of fusion protein expression

A Western immunoblotting technique was used to screen the fusion protein expression of each clone selected in the previous task.

An electrophoresis system was assembled and the running buffer (25mM Tris Base 3,02g; 192mM Glycine 14,42g; 0,1% SDS 1g; distilled water 1L) was prepared. The protein samples (supernatant collected after calcium transfection) were mixed with 4x Protein Loading buffer (10% (w/v) of SDS; 10% of beta-mercaptoethanol; 30% (w/v) of glycerol; 0.05% of bromophenol blue and 240mM Tris-HCl pH 6.8), denatured for 10 minutes at 95ºC and separated by 10% SDS-polyacrylamide gel. After running the gel at 20mA for approximately 100minutes, the system was tuned off, disassembled, and the gel was removed (stacking gel was cut off and discarded) to transfer the proteins to a nitrocellulose membrane.

To do so a wet transfer protocol was performed: two sheets of Whatman 3MM filter paper and one sheet of transfer nitrocellulose membrane were cut to the size of the gel and immersed in the transfer buffer (25mM Tris Base 3,02g; 192mM Glycine 14,42g; 20% methanol 200mL; dH_2O 1L). The transfer "sandwich" was assembled by placing, from the negative electrode to the positive electrode, the following items: sponge; filter paper sheet; SDS gel; nitrocellulose membrane; filter paper sheet; sponge. After ensuring that no air bubbles have been formed between the gel and membrane, the sandwich cassette was closed, the sandwich was placed in the transfer tank (containing the transfer buffer) with the membrane side closest to the positive electrode (red lead) and the running conditions were settled (400mA for 90 minutes). To verify the success of the wet transfer, the membrane was stained with Ponceau Red buffer for 10 minutes.

After washing the membrane with TBS buffer (50mM Tris-HCl, pH 7.4, 150mM NaCl) containing 0,1% of Tween 20 for 10 minutes, the blocking solution (TBS-0,1%T containing 3% nonfat dry milk) was added and the membrane was incubated with continuous agitation overnight at 4ºC to prevent non-

specific background binding. An anti-IgG antibody conjugated with HorseRadish Peroxidase (HRP) was added to the membrane (dilution 1:1000 in blocking solution) and incubated for 3 hours at room temperature with continuous agitation. The membrane was washed 3x 15 minutes with TBS-0,1%T and the immobilon western chemiluminescent HRP substrate was added and incubated for 5 minutes. The detection of antibody binding was done using chemiluminescence high sensitive light (ChemiDoc – BioRad).

The clones confirmed to be expressing the fusion protein were sent for sequencing (at Macrogen) to assure that the DNA sequence was correct.

4. Results

4.1. Screening of serum from patients with Myasthenia Gravis for anti-MIR peptide IgG antibodies

Patients with MG and anti-AchR antibodies (seropositive patients) do not have titters of antibodies that recognize the WNPDDYGGVK synthetic peptide significantly different from those detected in healthy controls or seronegative patients (Figure 5).

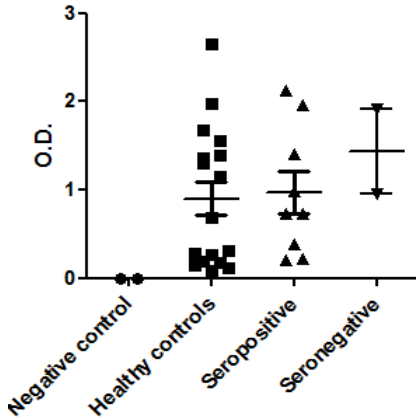

Figure 5 – Optical Density values of Myasthenia Gravis patients and controls when tested for the presence of anti-MIR peptide antibodies by ELISA assay

4.2. Identification and characterization of the B lymphocyte sub-population responsible for the production of autoantibodies in patients with Myasthenia Gravis

The peripheral blood mononuclear cells (PBMCs) isolated from the peripheral blood samples of both healthy subjects and patients with MG was used in this task (isolation procedure described above in Peripheral Blood Mononuclear Cells isolation). The samples from patients with MG treated with Rituximab were collected after 3 month of the last administration to eliminate possible bias associated with the drug regarding the circulating B lymphocytes.

a) Anti-human CD20 antibody APC-conjugated

In this task it was aimed to detect the CD20 membrane associated phosphoprotein that is present in the surface of every B-cell (except stem cells, pro-B-cells and effector B-cells) in order to distinguish the plasma cells involved in the production of the pathogenic antibodies: long-term plasma cells (CD20-) and short-term plasma cells (CD20+).

An increase in the concentration of the anti-human CD20 antibody APC-conjugated (Invitrogen) was proportional to the mean fluorescent intensity (MFI) detected in each sample (Figure 6).

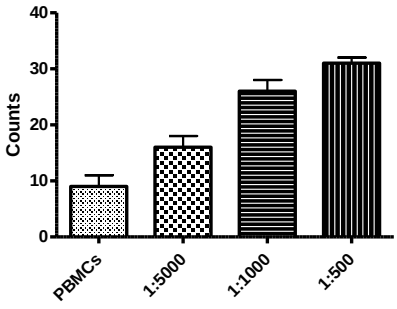

Figure 6 – Dilutions tested for anti-human CD20 antibody APC-conjugated

Comparison of the three dilutions tested for B lymphocyte detection by probing with anti-CD20 antibody. A control sample (without the probe) was used to determine the baseline fluorescence.

b) MIR synthetic peptide FICT-conjugated

The optimal condition of the MIR synthetic peptide was determined using only PBMCs samples from patients since it was not expected that this peptide would be recognized by B-cells from healthy controls.

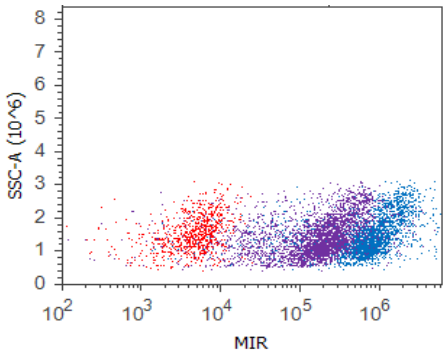

Figure 7 - Dilutions tested for MIR synthetic peptide FICT-conjugated

Dot plot graphic of the dilutions tested to detect B lymphocytes that recognized the MIR peptide. A control sample (without the probe) was used to determine the baseline fluorescence that is not due to the probe used in the MIR synthetic peptide (represented in red); in purple is represented the 1:100 dilution of the MIR peptide and in blue is the 1:50 dilution.

The lymphocytes present in the sample of one patient with MG recognized the MIR synthetic peptide in a concentration dependent fashion (Figure 7).

c) CD20/MIR (double staining)

In order to characterize the B lymphocytes responsible for the production of these antibodies we double stained the PBMCs samples from patients and healthy controls with the MIR synthetic peptide and anti-CD20 antibody.

The gates were defined considering the lymphocyte cell population and controls without probes were included in every assay. There were no differences between seropositive patients (patients whose

autoantibodies recognize the AchR) and seronegative patients and furthermore, the data showed that the lymphocyte population selected from the healthy controls was also positive for the MIR peptide with the majority of the cells being positive for the MIR peptide suggesting that the recognition of the MIR peptide was unspecific (Figure 8).

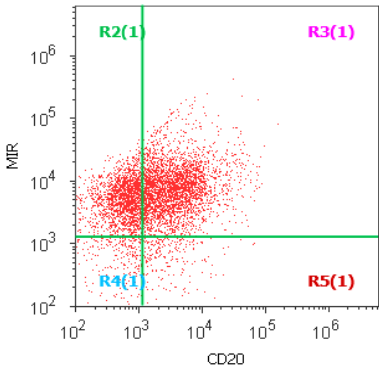

Figure 8 – CD20/MIR double staining in a healthy control sample

4.3. Development of a protein composed of the extracellular domain of the alpha 1 subunit of the AchR

4.3.1. Cloning 1 – CHRNA1 protein

4.3.1.1. Digestion – *pT7* vector

The *pT7* vector was digested using the *SfiI* restriction enzyme and separated in a 1% agarose gel and the results were visualized and photographed in UV light (ChemiDoc - BioRad):

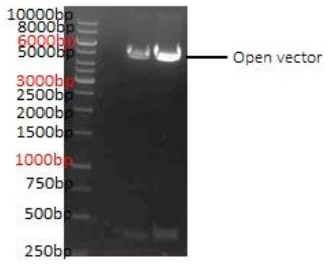

Figure 9 – *pT7* vector digestion with *SfiI* restriction enzyme

This agarose gel image shows the open *pT7* vector with a molecular weight of ≈5000bp.

The vector used in this task had approximately 5000bp and after the digestion with *SfiI* restriction enzyme was possible to see a band at a higher molecular weight corresponding to the opened vector and a band at a lower molecular weight (below the 500bp ladder mark) corresponding to segment that was excised with the digestion of the vector (Figure 9).

4.3.1.2. Digestion – CHRNA1 insert

The extracellular domain of the alpha 1 subunit of the nicotinic acetylcholine (CHRNA1) receptor was commercially synthesised and cloned into a *pMA* vector (a plasmid with ≈3000bp). Figure 10 shows the agarose gel where the DNA of interest was separated from its vector after the digestion with the same restriction enzymes as the vector in the previous task (*EcoRI* and *NcoI*). The band at the bottom of the agarose gel (approximately 681bp) matches the molecular weight of the DNA of interest (accordingly to the document provided by LifeTechnologies containing the plasmid information). Separated from the region of interest, at the top of the agarose gel with higher molecular weight (≈2400bp), is the open plasmid band.

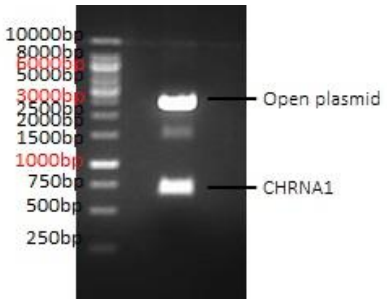

Figure 10 – CHRNA1 digestion with *EcoRI* and *NcoI* restriction enzymes

This agarose gel image shows the CHRNA1 band at the bottom of the image (on the right side) with a lower molecular weight (≈681bp) and a band at approximately 2400bp corresponding to opened plasmid into which the CHRNA1 was cloned at Life technologies in vector's digestion.

4.3.1.2.1. CHRNA1 for *pT7* vector

The gene of interest (CHRNA1) digested in the previous step was amplified with specific primers, that include the restriction sites for *SfiI* enzyme. In this reaction a negative control (without template DNA) was used.

The agarose gel image obtained after the DNA amplification showed that in the positive reactions (with the template DNA) there was a brighter band, near the 750bp ladder band, that corresponded to the CHRNA1 molecular weight (681bp) plus the two primers, making the final molecular weight slightly higher than the CHRNA1 gene alone. It is also possible to see a band at 500bp that might indicate unspecific binding or an incomplete amplification and for that reason these bands were not excised for DNA purification. Additionally, a band with a significant lower weight than 250bp was present in every well (either in the positive or in the negative) which could represent the unbounded primers

used for CHRNA1 gene amplification, since in the negative control this was the only band that appeared in the agarose gel (Figure 11).

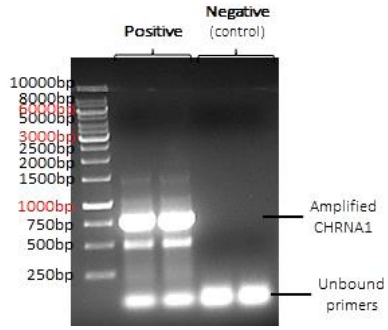

Figure 11 – Amplified CHRNA1 DNA using specific primers

In the positive wells was possible to see the amplified CHRNA1 DNA and in both positive and negative control wells the band at the bottom of the image corresponds to the primer used in this task that did not bound to the CHRNA1 DNA.

4.3.1.3. Screening of CHRNA1-*pT7* binding

After the binding of the open *pT7* vector to the amplified CHRNA1 with *SfiI* restriction sites, the resulting DNA was transformed into JM109 *E. coli* bacterial strains and plated in an ampicillin selective LB-agar plate. The plates used for bacterial colonies growth were: A) plate with the DNA/Vector ratio 3:1 and B) negative control (without CHRNA1 DNA). Twenty seven bacterial colonies were chosen for screening by PCR and the DNA isolated was screened using a similar protocol as in CHRNA1 DNA amplification (see CHRNA1 for *pT7* vector) and then separated in an agarose gel.

Considering that the screening by PCR was a procedure similar to the one already used, it was expected that the results obtained were similar to the ones before.

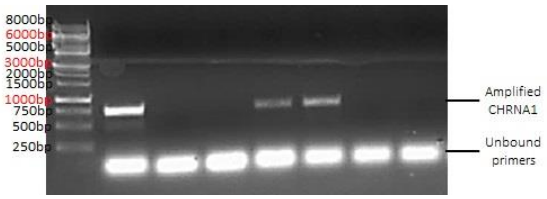

Figure 12 – Screening of CHRNA1-*pT7* binding

This agarose gel represents part of the tested colonies for CHRNA1-*pT7* binding where it was possible to see that only three of seven tested colonies in this gel were positive for the CHRNA1 DNA.

Some of the bacterial colonies selected for screening had the CHRNA1 DNA integrated in the vector (Figure 12).In the presence of specific primers, the CHRNA1 DNA was amplified obtaining an analogous image as to the one shown in Figure 11: a band near the 750bp ladder band that corresponded to the

CHRNA1 molecular weight (681bp) plus the two primers used and a band of the unbounded primers at a very low molecular weight (under 250bp).

From the 27 tested colonies for CHRNA1-*pT7* binding only seven were considered positive and chosen for protein expression.

4.3.1.4. Screening of CHRNA1 protein expression assay

The screening for the CHRNA1 protein ((MW± 30kDa) was performed by two different procedures: an ELISA and a western blot. The first assay was used to assess the protein expression while the western blot was used to confirm if the protein expressed had the correct molecular weight. The optical densities (OD) obtained with the ELISA method (data not shown) revealed that all clones tested expressed the CHRNA1 protein. The disparity in the optical densities suggested differences in the expression rates between clones however these data by themselves were not definitive, requiring another technique to confirm them.

The western blot was performed using x-ray film exposure. This technique rapidly exposes the film to saturation allowing the detection of weakly expressed proteins that would not be detected in chemiluminescent imaging.

Four of the clones tested are represented in Figure 13 in which the negative control (second well to the left) had no protein expression and the positive control expressed the 35kDa protein as expected.

The clones were considered positive if after revelation and imaging was possible to see a clear band at the correct molecular weight. Only two of the clones tested for the CHRNA1 protein expression were considered to be positive and sent for sequencing in order to confirm that the DNA sequence was exactly the same as the one from the original gene of CHNRA1.

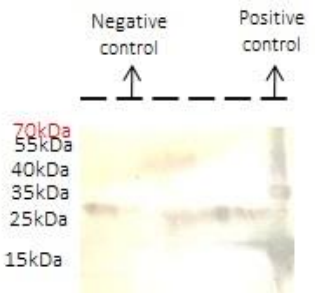

Figure 13 – Screening of CHRNA1 protein expression in the selected clones by western blot

These western blot film shows that only one of the four tested clones here expressed the CHRNA1 protein. Both negative (without IPTG induction) and positive (protein expressed at 35kDa) controls were included.

The fact that there was the need to resort to a much more sensitive imaging technique to detect the protein bands suggests that the conditions used in the protein expression assay were not the best ones.

4.3.1.4.1. Purification of CHRNA1 protein

After confirmation of correct sequence we began the purification by affinity chromatography following a standard protocol. The CHRNA1 protein was not isolated from other proteins present in the bacterial culture, and these were being eluted at the same time, suggesting that the buffers used for binding and elution were not effective and the CHRNA1 protein did not bound to the Nickel-Sepharose beads of the HIS-Trap columns.

4.3.2. Measurement of anti-CHRNA1 IgG antibodies

Although the CHRNA1 protein had not been purified, it would still be the proposed target for the autoantibodies associated with MG. Therefore, we assessed the serum from patients with MG for antibodies that would recognize the protein. Samples with OD values of 3 standard deviations (SD) above the mean of the healthy controls were considered to be positive.

We found a significant difference between the titres of anti-CHRNA1 protein in patients when compared with controls (p value=0,031). Furthermore, ≈50% of the patients with anti-AchR antibodies (seropositive patients) were positive for antibodies that recognized the CHRNA1 protein (Figure 14).

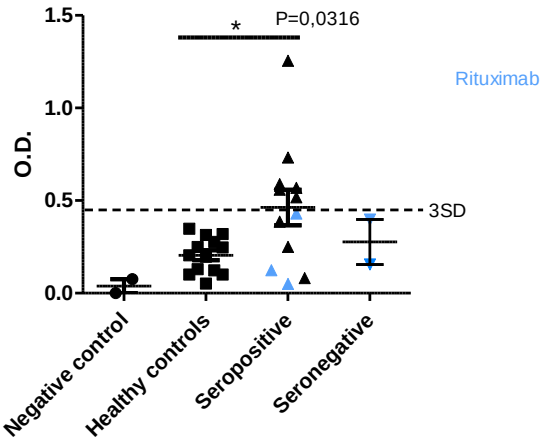

Figure 14 - Optical Density values of patients with Myasthenia Gravis and controls when tested for the presence of anti-CHRNA1 antibodies by an ELISA assay

50% of the patients tested recognized the CHRNA1 protein (antibodies titres cut-off defined by mean) (p value=0,0316). Patients highlighted in blue besides the immunosuppressive therapy were also treated with Rituximab.

All patients enrolled in this study were being treated with corticosteroids and immunosuppressive drugs, such as prednisolone and azathioprine. However some patients were also treated with Rituximab. The patients treated with Rituximab had lower values of anti-CHRNA1 antibodies (p value=0,1) (Figure 15). This finding can be explained as Rituximab is extremely effective in depleting the circulating B lymphocytes and therefore these patients could have a decrease in the production of the antibodies. Nevertheless, the number of patients collected in this group is very small and therefore, the interpretation of these results should be careful.

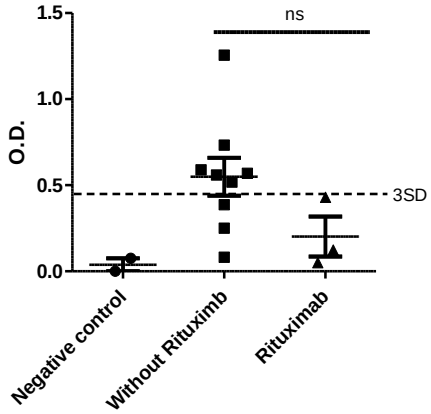

Figure 15 - Optical Density values of seropositive patients with MG treated with and without Rituximab when tested for the presence of anti-CHRNA1 antibodies by an ELISA assay
The seropositive patients treated with Rituximab had lower values of anti-CHRNA1 antibodies (p value=0,1).

4.4. Development of a fusion protein composed of the extracellular domain of the alpha 1 subunit of the AchR and the Fc region of a immunoglobulin

4.4.1. Cloning 2 – fusion protein

4.4.1.1. Digestion – *pFUSE-hIgG1e1-Fc2* Vector

The *pFUSE-hIgG1e1-Fc2* vector was digested using the restriction enzymes *EcoRI* and *NcoI*. The results were visualized and photographed in UV light (ChemiDoc - BioRad) (Figure 16).

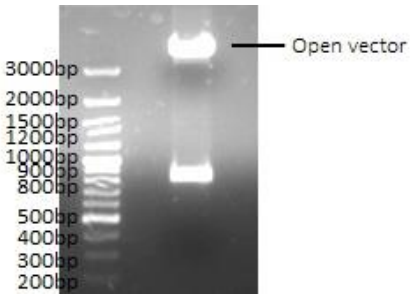

Figure 16 – *pFUSE-hIgG1e1-Fc2* vector digestion with *EcoRI* and *NcoI* restriction enzymes

Agarose gel image that shows the vector with a higher molecular weight (4197bp) above the ladder band at 3000b, and a band at approximately 900bp corresponding to region that was excised in vector's digestion.

This figure shows a band matching the molecular weight of the vector at the top of the agarose gel, since the vector has 4197bp, which was separated from the region excised when the vector was digested with the restriction enzymes *EcoRI* and *NcoI* (band at approximately 900bp).

4.4.1.2. Screening of CHRNA1-*pFUSE-hIgG1e1-Fc2* binding

The opened vector and the CHRNA1 DNA were fused together with a specific enzyme (T4 DNA ligase), transformed into JM109 bacterial cells and plated in an antibiotic selective LB-agar plates: the plate A (DNA/Vector ratio 3:1) had several isolated colonies; plate B (DNA/Vector ratio 5:1) had small bacterial colonies filling the plate, making impossible to isolate one colony; the negative control (plate C) had no colonies. The plate selected to screen the CHRNA1-*pFUSE-hIgG1e1-Fc2* binding was the one with the DNA/Vector ratio of 3:1. Fourteen of those colonies were used for alkaline lysis and the DNA isolated was digested using the same protocol as in *Digestion – pFUSE-hIgG1e1-Fc2* Vector and then separated in an agarose gel.

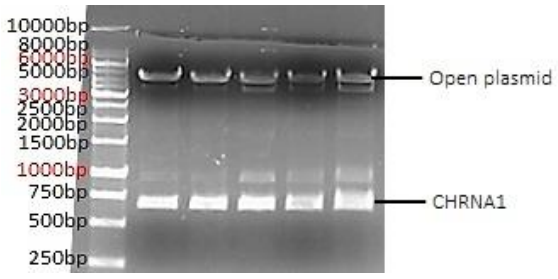

Figure 17 – Screening of CHRNA1–*pFUSE-hIgG1e1-Fc2* binding

In this agarose gel was possible to see some of the colonies tested for the CHRNA1 - *pFUSE-hIgG1e1-Fc2* binding. Beneath the ladder band that corresponds to the 5000bp molecular weight it was possible to observe one band in each well (matching the *pFUSE-hIgG1e1-Fc2* vector weight) and at the bottom the CHRNA1 band with a lower molecular weight (≈681bp).

Some of the colonies tested and separated in the agarose gel are represented in Figure 17. The *pFUSE-hIgG1e1-Fc2* vector band appeared in every tested bacterial clone slightly below the ladder band that

marks the molecular weight of 5000bp, since the vector has 4197bp.The bottom of the gel shows the bands matching the molecular weight of the CHRNA1 (≈681bp). In some wells, such as the third and fifth wells of this gel, it was possible to see two extra bands: one at approximately at 1000bp of molecular weight and another one underneath the band that corresponds to the digested vector, these bands may indicate a non-completed digestion of the DNA isolated in the alkaline lysis task or an incorrect binding of CHRNA1 DNA to the *pFUSE-hIgG1e1-Fc2* vector thus these clones were not chosen for the protein expression assays.

4.4.1.3. Screening of fusion protein expression

The previous task allowed the selection of six clones to express the fusion protein using calcium phosphate transfection after which the supernatant was collected and the protein expression was assessed by Western Blot.

The fusion protein was expected to have approximately 55kDa (the molecular weight of the CHRNA1 protein – ≈30kDa – plus the Fc domain of the immunoglobulin), hence the positive control used was a DNA that expresses a protein with the same molecular weight as the tested fusion protein. Additionally, a negative control (without DNA) was included in the transfection to rule out the possibility that another protein could be expressed or present in the supernatant with the same molecular weight as the fusion protein.

Some of the clones tested are represented in the figure below (Figure 18): the two positive controls expressed the protein at 55kDa and the negative control did not express any protein as was expected. The image shows that only one of the four clones tested expressed the fusion protein and did so at a very low rate.

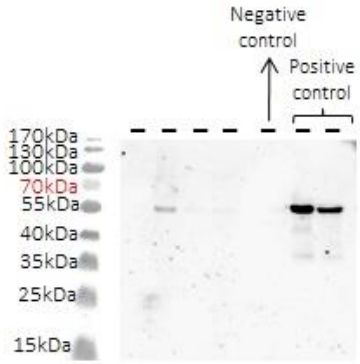

Figure 18 – Screening of fusion protein expression in selected clones

These western blot results showed that only one of the four tested clones here expresses the fusion protein. Negative (without DNA) and positive (DNA known to be expressing a protein with the same molecular weight as the fusion protein tested) controls were included.

Three clones confirmed to be expressing the fusion protein and were sent for sequencing to confirm if the DNA sequence was the correct one.

5. Discussion

The MIR peptide was described as the major antigen in an animal model of Myasthenia Gravis (EAMG). However its relevance in humans had never been tested. We investigated whether this peptide would be significant in human patients with MG. Our results showed that the anti-MIR peptide antibodies titres in serum from seropositive patients with MG were no different from those found in seronegative patients or healthy controls, which suggests that the MIR peptide might not be the main immunogenic region targeted by autoantibodies in human MG.

We then decided to confirm these results using a different method and used flow cytometry to determine whether these patients had CD20 B-Lymphocytes that would express the anti-MIR membrane immunoglobulins. This second set of experiments reinforced the results previously obtained in which the MIR peptide had not been recognized by the autoantibodies as a main target in patients with Myasthenia Gravis, contrarily to what was reported in animal models of this disease. We find these results very important as they highlight the limitations that the use of these models might have when attempting to study the mechanisms of human MG.

Consequently, it became essential to identify other antigenic regions in the nicotinic acetylcholine receptor that could be recognized by the autoantibodies produced in these patients. The alpha1 subunit of the AchR is its most functionally important subunit, and therefore a potential prime target for the immune system that would explain the strong relation between antibody presence and receptor dysfunction. Part of this work was directed into synthesising the extracellular domain of this subunit as a first attempt to narrow down the antigen site within the receptor.

The CHRNA1 protein purification was a major time consuming setback in this project and despite our continuous attempts it was not possible to purify it.

However, with this work it was possible to confirm our hypothesis that the alpha1 subunit, being the receptor's subunit which is more biologically active, is an important target for antibody binding as ≈50% of the autoantibodies from patients with Myasthenia Gravis recognized the CHRNA1 cloned protein. Interestingly, these results suggest that other antigenic regions in the nicotinic AchR besides the alpha1 subunit should be considered.

During this project, we also produced a tool with the aim of identifying the autoantibody-producing B-cells in patients with MG: a fusion protein constituted by the extracellular domain of the alpha1 subunit of the AchR (CHRNA1), and the Fc region of an IgG immunoglobulin. Even though most of this task has been completed, time limitations did not allow the complete purification of this fusion protein.

Combined together, the results presented here, although preliminary, are very promising in redirecting the search for other antigenic targets in the AchR.

6. Conclusions

The work presented in this thesis is an important step towards determining the specific antigenic targets of autoantibodies that bind to the acetylcholine receptor in patients with MG.

We were able to create tools that enable the screening of the serum of patients with MG for the presence of more specific autoantibodies that recognize either the MIR peptide or the alpha1 subunit of the AchR, and that may allow the identification of the autoantibody-producing B-cells in these patients.

It is now possible to infer that the MIR peptide described to be the main immunogenic region in antibody targeting in animal models of the disease (EAMG), may not be the same as in humans. Moreover, we showed that the serum from seropositive patients with MG had antibodies directed towards the CHRNA1 suggesting that this subunit is an important target of antibody binding in MG.

Adding to the fact that these results may lead to the creation of a more specific tool for the diagnosis and clinical monitoring of the disease, the identification of CD20 cells as the lymphocytes responsible for the production of the pathogenic antibodies would allow the possibility of using specific anti-CD20 drugs (such as rituximab) in the overall treatment of the disease or in specific subsets of patients, defined by the type of antibodies they would carry.

Furthermore, the finding of the antibody's specific target may result in the development of a fusion protein capable of binding to anti-AchR autoantibodies and subsequently blocking their linkage to the AchR, creating in this fashion a new class of drugs for the treatment of this disease.

7. Future work

The work presented here is still a preliminary work and further studies are needed. The localization, structure and antigenic properties of the binding site of the antibodies to the acetylcholine receptor, and the full comprehension and characterization of both B lymphocytes and immunoglobulins involved in the pathogenicity of this disease are crucial to the development of new biomarkers and new drugsfor Myasthenia Gravis.

Due to unexpected obstacles during this project's development it was not possible to purify the proteins produced by cloning, nevertheless in future work we intend to successfully perform this task and create a vast panel of peptides to test antibody binding and determine the specific area that is targeted by the autoantibodies. Furthermore we aim to determine whether CD20+ B lymphocytes are responsible for the production of these pathogenic antibodies.

The use of the tolls developed in (or following) this project may be decisive in the implementation of new diagnostic and therapeutic strategies leading to a personalized and more efficient medical care of these patients.

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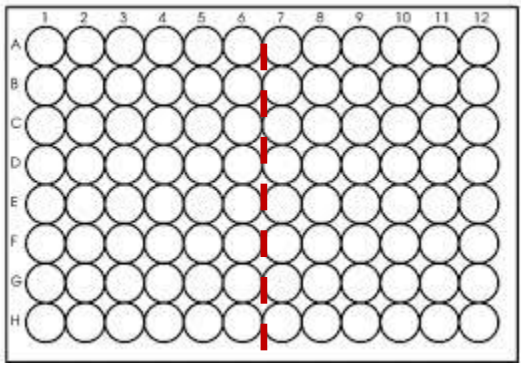
9. Appendix

Appendix 1 – ELISA method



1,5 µg/mL of synthetic MIR peptide

5 µg/mL of CHRNA1 protein

Half-coated

Half-uncoated

Incubate overnight at 4ºC

Blocking buffer

Incubate 1h at 37ºC

Washing step

Add serum samples

Incubate 1h at 37ºC

Washing step

Add Conjugated antibody

Incubate 1h at 37ºC

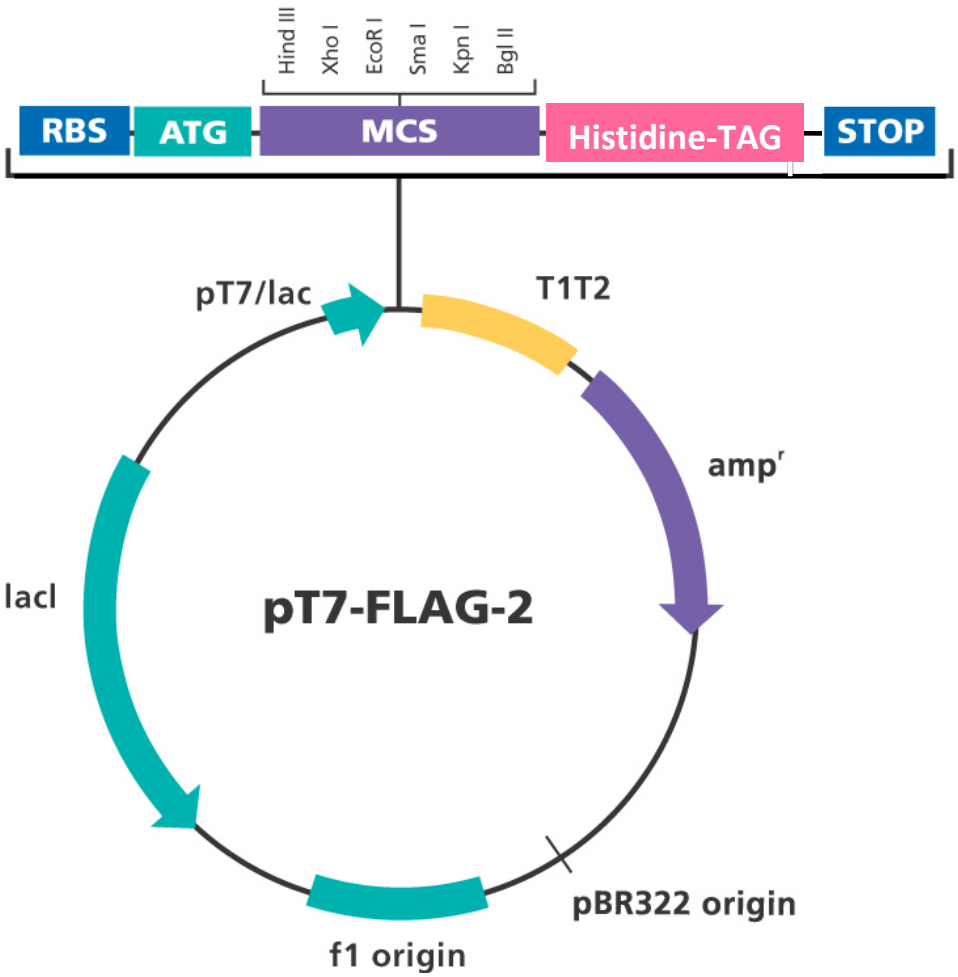
Washing step

Add specific substrate

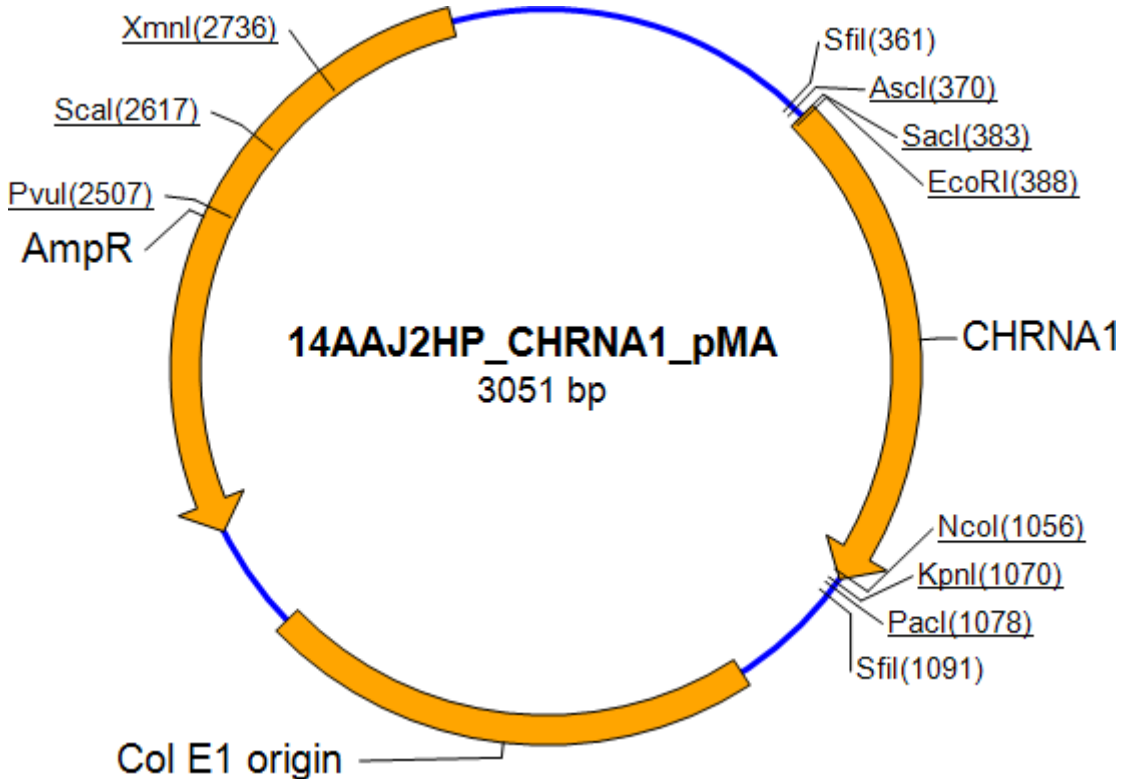
Incubate 1h at 37ºC

Read optical density at 495nm with reference to 605nm

Appendix 2 – *pT7* vector



Appendix 3 – synthetic gene CHRNA1



Appendix 4 – *pFuse-hIgG1e1-Fc2* vector